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## UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 C.F.R. § 1.53(b))

Attorney Docket No. 600-1-182NA

First Inventor or Application Identifier Vinkemeier

Title PURIFIED STAT PROTEINS AND ...

Express Mail Label No. EL406398175US

### APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1.  \* Fee Transmittal Form (e.g., PTO/SB/17)  
(Submit an original and a duplicate for fee processing)
2.  Specification [Total Pages 96]
  - Descriptive title of the Invention
  - Cross References to Related Applications
  - Statement Regarding Fed sponsored R & D
  - Reference to Microfiche Appendix
  - Background of the Invention
  - Brief Summary of the Invention
  - Brief Description of the Drawings (if filed)
  - Detailed Description
  - Claim(s)
  - Abstract of the Disclosure
3.  Drawing(s) (35 U.S.C. 113) [Total Sheets 14]
4. Oath or Declaration [Total Pages 3]
  - a.  Newly executed (original or copy)
  - b.  Copy from a prior application (37 C.F.R. § 1.63(d))  
(for continuation/divisional with Box 16 completed)
    - i.  DELETION OF INVENTOR(S)  
Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. §§ 1.63(d)(2) and 1.33(b).

\*NOTE FOR ITEMS 1 & 13: IN ORDER TO BE ENTITLED TO PAY SMALL ENTITY FEES, A SMALL ENTITY STATEMENT IS REQUIRED (37 C.F.R. § 1.27), EXCEPT IF ONE FILED IN A PRIOR APPLICATION IS RELIED UPON (37 C.F.R. § 1.28).

ADDRESS TO: Assistant Commissioner for Patents  
Box Patent Application  
Washington, DC 20231

5.  Microfiche Computer Program (Appendix)
6. Nucleotide and/or Amino Acid Sequence Submission  
(if applicable, all necessary)
  - a.  Computer Readable Copy
  - b.  Paper Copy (identical to computer copy)
  - c.  Statement verifying identity of above copies

### ACCOMPANYING APPLICATION PARTS

7.  Assignment Papers (cover sheet & document(s))
8.  37 C.F.R. § 3.73(b) Statement  Power of  
(when there is an assignee)  Attorney
9.  English Translation Document (if applicable)
10.  Information Disclosure Statement (IDS)/PTO-1449  Copies of IDS  
Citations
11.  Preliminary Amendment
12.  Return Receipt Postcard (MPEP 503)  
(Should be specifically itemized)
13.  Small Entity Statement(s)  Statement filed in prior application,  
(PTO/SB/09-12) Status still proper and desired
14.  Certified Copy of Priority Document(s)  
(if foreign priority is claimed)
15.  Other: Request for Transfer of Computer  
Readable Form; new IDS; and  
new Format Drawings

16. If a CONTINUING APPLICATION, check appropriate box, and supply the requisite information below and in a preliminary amendment:

Continuation  Divisional  Continuation-in-part (CIP)

of prior application No: 08/951,130

Group / Art Unit: 1636

Prior application information: Examiner YUCEL

For CONTINUATION or DIVISIONAL APPS only: The entire disclosure of the prior application, from which an oath or declaration is supplied under Box 4b, is considered a part of the disclosure of the accompanying continuation or divisional application and is hereby incorporated by reference. The incorporation can only be relied upon when a portion has been inadvertently omitted from the submitted application parts.

### 17. CORRESPONDENCE ADDRESS

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT : UWE VINKEMEIER *ET AL.*

SERIAL NO. : UNASSIGNED EXAMINER : UNKNOWN

FILED : HEREWITH ART UNIT : UNKNOWN

FOR : PURIFIED STAT PROTEINS AND METHODS OF PURIFYING THEREOF

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS  
BOX PATENT APPLICATION  
WASHINGTON, DC 20231

Sir:

In accordance with Rule 111 of the Rules of Practice please consider the following amendments and remarks.

Please amend the above-identified Application as follows:

IN THE SPECIFICATION:

On Page 1, line 11 after "is" please insert - - a Continuation of copending U.S. Serial No. 08/951,130 filed on October 15, 1997 which is - - therefor.

On line 12, after "the" please replace

"the disclosure of which is hereby incorporated by reference in its entirety. Applicants claim the benefits of this Application under 35 U.S.C. § 119(e)." with

- - the disclosures of which are hereby incorporated by reference in their entireties. Applicants claim the benefits of these Application under 35 U.S.C. §§ 119 (e) and 120. - - therefor.

On Page 15, line 6, please replace "Figure 2B." with - - Figures 2B-2D - - therefor; and

on line 15, after "Figure" please replace "2C" with - - 2E - - therefor.

On Page 16, line 4, please replace "Figure 4" with - - Figures 4A and 4B - - therefor;  
on line 13, please replace "Figure 5A" with - - Figures 5A and 5B - - therefor;  
and on line 24, please replace "Figure 5B" with - - Figures 5C and 5D - - therefor.

On Page 45, line 23, after "autoradiography." please replace "Plagues" with  
- - Plaques - -, therefor.

On Page 55, line 15, after "5'-dGGGAATTCCATATGAGCACAGTGATG-TTAGACAAAC" please insert - - (SEQ ID NO:7) - -, therefor;  
and on line 16, after "5'-dCGGATCCTATTAGTGAACTTCAGACACAGAAATC" please insert - - (SEQ ID NO:8) - -, therefor.

On Page 59, line 32 after "5'-dGTATTCCCGTCAATGCA-3'" please insert - - (SEQ ID NO:9) - -, therefor, and

after "5'-dGTATTCCGTAAAGATCT-3'" please insert - - (SEQ ID NO:10) - -, therefor;  
on line 33 after "5'-dGATTCCCGTAAATCAT-3'" please insert - - (SEQ ID NO:11) - -, therefor, and  
after "5'-dGTTGTTCCGGAAAAGG-3'" please insert - - (SEQ ID NO:12) - -, therefor;  
on line 34  
after "5'-dAGTCAGTTCCCGTCAATGCATCAGGTTCCCGTCAATGCAT-3'" please insert - - (SEQ ID NO:13) - -, therefor.

On Page 60, line 1,  
after "5'-dAGTCAGTTCCCGTCAATGAGTTCCCGTCAATGCA-3'" please insert  
- - (SEQ ID NO:14) - -, therefor;  
on line 3

after "5'-dAGTCAGTTCCCGTCAATGATCGCTACAGAGTTCCGTCAAGCA-3"

please insert - - (SEQ ID NO:15) - -, therefor; and

on line 5

after "5'-dAGTCATTTCCGTCAATGCATCAGTTGACGGGAAAGTAGT-3"

please insert - - (SEQ ID NO:16) - -, therefor.

on line 13, after "Figures" please replace "5B" with - - 5C-5D - - therefor.

On Page 66, on line 13, after "Figure" please replace "2B" with - - 2B-2D - - therefor;

on line 15, after "Figure" please replace "2B" with - - 2B-2D - - therefor;

on line 23, after "Figure" please replace "2C" with - - 2E - - therefor;

on line 26, after "Figure" please replace "2C" with - - 2E - - therefor; and

on line 28, after "Figure" please replace "2C" with - - 2E - - therefor.

On Page 67, line 13, please replace "Figure 4" with - - Figures 4A and 4B - - therefor.

On Page 68, line 2, please replace "Figure 5B" with - - Figure 5C - - therefor.

Please insert the accompanying Sequence Listing in its appropriate place in the Specification.

IN THE CLAIMS:

Please cancel Claims 2-55 without prejudice.

Please add the following new claims:

- - 56. A method for identifying a drug that modulates the ability of adjacent STAT protein dimers to interact comprising measuring the ability of a test compound to modulate the association of a first STAT protein or a fragment of said first STAT protein with a second STAT

protein or a fragment of said second STAT protein;

wherein said fragment of said first STAT protein comprises the N-terminal domain of said first STAT protein;

wherein said fragment of said second STAT protein comprises the N-terminal domain of said second STAT protein;

wherein the association is dependent upon the N-terminal domain of said first STAT protein, and the N-terminal domain of said second STAT protein; and

wherein a test compound which enhances the association is identified as a drug that enhances the interaction between adjacent activated STAT dimers, whereas a test compound that decreases the association is identified as a drug that inhibits the interaction between adjacent activated STAT dimers.

57. The method of Claim 56 wherein said first STAT protein is selected from the group consisting of STAT 1, STAT 2, STAT 3, STAT 4, STAT 5A, STAT 5B, and STAT 6.

58. The method of Claim 56 wherein said second STAT protein is selected from the group consisting of STAT 1, STAT 2, STAT 3, STAT 4, STAT 5A, STAT 5B, and STAT 6.

59. The method of Claim 56 wherein said first STAT protein and said second STAT protein are the same STAT protein.

60. A method for identifying a drug that modulates the ability of adjacent STAT protein dimers to interact and bind to adjacent DNA binding sites comprising:

- (a) determining the ability of a STAT protein or a fragment of the STAT protein to bind to a nucleic acid comprising two adjacent weak STAT DNA binding sites in the presence and absence of a test compound;
- (b) determining the ability of the STAT protein or fragment of the STAT protein to bind to a nucleic acid comprising a single strong STAT DNA binding site in the

presence and absence of a test compound;

wherein said STAT protein fragment comprises the N-terminal domain of said STAT protein; and wherein a test compound that increases binding in step (a) but not in step (b) is identified as a drug that enhances the interaction between adjacent activated STAT dimers, and a test compound that decreases binding in step (a) but not in step (b) is identified as a drug that inhibits the interaction between adjacent activated STAT dimers.

61. The method of Claim 60 wherein said STAT protein is selected from the group consisting of STAT 1, STAT 2, STAT 3, STAT 4, STAT 5A, STAT 5B, and STAT 6.

62. A method for identifying a drug that modulates the ability of adjacent STAT protein dimers to interact comprising measuring the ability of a test compound to modulate the association of a fragment of a first STAT protein with a second STAT protein or a fragment of said second STAT protein dimer;

wherein said fragment of said first STAT protein consists essentially of the N-terminal domain of said first STAT protein;

wherein said fragment of said second STAT protein comprises the N-terminal domain of said second STAT protein;

wherein the association is dependent upon the N-terminal domain of said first STAT protein, and the N-terminal domain of said second STAT protein; and

wherein a test compound which enhances the association is identified as a drug that enhances the interaction between adjacent activated STAT dimers, whereas a test compound that decreases the association is identified as a drug that inhibits the interaction between adjacent activated STAT dimers.

63. The method of Claim 62 wherein said first STAT protein is selected from the group consisting of STAT 1, STAT 2, STAT 3, STAT 4, STAT 5A, STAT 5B, and STAT 6.

64. The method of Claim 62 wherein said second STAT protein is selected from the group consisting of STAT 1, STAT 2, STAT 3, STAT 4, STAT 5A, STAT 5B, and STAT 6.

65. The method of Claim 62 wherein said first STAT protein and said second STAT protein are the same STAT protein. - -

REMARKS

The amendments to the Specification were made to correct obvious typographical errors, to conform the Specification with the formal drawings (copies of which are enclosed), and/or to conform the sequences contained in the original Specification with the Sequence Listing (enclosed).

The Applicants have canceled Claims 2-55 without prejudice in favor of prosecuting Claim 1, and newly added Claims 56 - 65. Support for newly added Claims 56-65 can be found throughout the Specification as originally filed including in the original claims. Further support for Claim 56 can be found on line 10 of Page 12 through line 9 of Page 13, and on line 14 of Page 50 through line 12 of Page 51. Further support for Claims 57-59, 61, and 63-65 can be found on Page 18, lines 16-19, Page 19, lines 17 and 18, Page 70 lines 10 and 11, and Page 71, lines 27-29. Further support for Claim 60 can be found on line 1 of Page 11 through line 8 of Page 12, and on Page 50, lines 14-28. Further support for Claim 62 can be found on line 10 of Page 12 through line 9 of Page 13, and on line 30 of Page 50 through line 12 of page 51. No new matter has been entered.

PATENT  
600-1-182N

Applicants respectfully request entry of the foregoing amendments into the file history of the above-identified Application being filed herewith. Early and favorable action on the pending Claims are earnestly solicited.

Respectfully submitted,

  
MICHAEL D. DAVIS  
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Date: November 2, 1999

**PURIFIED STAT PROTEINS AND METHODS OF PURIFYING THEREOF**

**GOVERNMENTAL SUPPORT**

- 5 The research leading to the present invention was supported, at least in part, by NIH Grant Nos. AI32489 and AI34420. Accordingly, the Government may have certain rights in the invention.

**CROSS-REFERENCE TO RELATED APPLICATIONS**

10

The present Application is based upon provisional application U.S. Serial No. 60/028,176, filed October 15, 1996, the disclosure of which is hereby incorporated by reference in its entirety. Applicants claim the benefits of this Application under 35 U.S.C. § 119(e).

15

**FIELD OF THE INVENTION**

The present invention relates generally to methods of purifying recombinant Stat proteins, modified Stat proteins and functional fragments thereof. Included in the present invention are the purified proteins and fragments themselves. The present invention also relates to  
20 methods of separating phosphorylated species of these proteins and fragments from the nonphosphorylated forms. The present invention also relates to methods for using purified Stat proteins, truncated Stat proteins or N-terminal fragments of Stat proteins for drug discovery.

25

**BACKGROUND OF THE INVENTION**

Transcription factors play a major role in cellular function by inducing the transcription of specific mRNAs. Transcription factors, in turn, are controlled by distinct signalling molecules. One particular family of transcription factor consists of the Signal Transducers  
30 and Activators of Transcription (Stat) proteins. Presently, there are seven known mammalian Stat family members. The recent discovery of Drosophila and Dictyostelium discoideum Stat proteins suggest that Stat proteins have played an important role in signal transduction since the early stages of our evolution [Yan R. *et al.*, *Cell* 84:421-430 (1996); Kawata *et al.*, *Cell* 89:909 (1997)].

35

Stat proteins mediate the action of a large group of signalling molecules including the

- cytokines and growth factors (Darnell *et al.* WO 95/08629, 1995). One distinctive characteristic of the Stat proteins are their apparent lack of requirement for changes in second messenger, *e.g.*, cAMP or Ca<sup>++</sup>, concentrations. Another characteristic is that Stat proteins are activated in the cell cytoplasm by phosphorylation on a single tyrosine
- 5 (Darnell *et al.*, 1994; Schindler and Darnell, 1995). The responsible kinases are either ligand-activated transmembrane receptors with intrinsic tyrosine kinase activity, such as EGF- or PDGF-receptors, or cytokine receptors that lack intrinsic kinase activity but have associated JAK kinases, such as those for interferons and interleukins (Ihle, 1995). When Stat proteins are phosphorylated, they form homo- or heterodimeric structures in which
- 10 the phosphotyrosine of one partner binds to the SRC homology domain (SH2) of the other. The newly formed dimer then translocates to the nucleus, binds to a palindromic GAS sequence, thereby activating transcription (Shuai *et al.*, 1994; Qureshi *et al.*, 1995; Leung *et al.*, 1996).
- 15 Stat proteins serve in the capacity as a direct messengers between the cytokine or growth factor receptor present on the cell surface, and the cell nucleus. However, since each cytokine and growth factor produce a specific cellular effect by activating a distinct set of genes, the means in which such a limited number of Stat proteins mediate this result remains a mystery. Indeed, at least thirty different ligand-receptor complexes signal the
- 20 nucleus through the seven known mammalian Stat proteins [Darnell *et al.*, *Science* 277:1630-1635 (1997)].
- Clearly there is a need to further study the biochemistry of Stat proteins. Unfortunately current studies are seriously hampered due to the low quantities of purified protein
- 25 available. Full-length cDNAs for all mammalian Stats have been cloned. In addition, certain Stat proteins have been expressed in baculovirus-infected insect cells using a His tag at the COOH-terminal end and then purified by Ni-affinity chromatography (Xu, X., *et al.*, *note 9* (1996)). However, no one has reported the production of milligram quantities of activated Stat protein, nor more importantly, a purification process amenable
- 30 to scaling up for such quantitative isolations.

To perform the biochemical studies necessary to understand the mechanism of the Stat-mediated signal transduction, and to configure assays useful for the detection of compounds that modulate Stat function, there remains an unfulfilled requirement for the

production of large amounts of pure protein. Furthermore, there is a need for a means of specifically phosphorylating the correct tyrosine residue on a Stat protein and then separating the resulting phosphorylated Stat protein from the unphosphorylated form in quantitative yields. In addition, there is a need to produce large quantities of stable,

- 5 soluble truncated Stat proteins that retain functional activities of the corresponding native Stat protein. Finally, there is a need to develop methods of isolating these functional truncated Stat proteins.

The citation of any reference herein should not be construed as an admission that such

- 10 reference is available as "Prior Art" to the instant application.

#### SUMMARY OF THE INVENTION

The present invention describes recombinant human Stat proteins which are produced in  
15 insect cells infected with recombinant baculovirus. Stable truncated forms of these proteins produced in bacteria are also included in the present invention. The present invention also includes labeled recombinant human Stat proteins and truncated Stat proteins. One aspect of this invention includes the purification of large amounts of these recombinant proteins. These isolated Stat proteins can be isolated in either their activated  
20 form, *i.e.*, having a phosphorylated tyrosine, or in the nonphosphorylated state, where the corresponding tyrosine residue is not phosphorylated. A related aspect to the invention details the protease sensitivity of Stat proteins and the important consequences of this particular property. The present invention exploits this property and describes a recombinant truncated Stat protein that can be expressed in a bacterial host in large  
25 quantities, as a soluble protein that can be readily purified by the teaching of the present invention. The phosphorylated and nonphosphorylated form of the truncated Stat protein can also be individually isolated.

The expression of the truncated protein in a soluble form overcomes earlier failures,  
30 where recombinant Stat proteins formed almost exclusively insoluble inclusion bodies. Other potentially active fragments of Stat proteins that contain the DNA binding domain, either form insoluble inclusion bodies or are themselves so susceptible to proteolysis that isolation of the large quantities necessary for biochemical studies are not practical. Thus the present invention teaches for the first time, a soluble recombinant truncated Stat

protein, as well as methods of its expression and isolation.

Although the present invention includes all Stat proteins, when specific amino acid residues are identified by number, the number represents the sequential position of that 5 amino acid in the amino acid sequence of Stat1 $\alpha$ . Thus, the number denoted for a specified amino acid in Stat1 $\beta$  and Stat1 $\gamma$ , as used herein, is per its corresponding position in the amino acid sequence of Stat1 $\alpha$ .

- The present invention includes a truncated Stat protein that can be expressed as a soluble 10 recombinant protein in a bacterial host cell. In preferred embodiments the bacterial host is *E. coli*, and the soluble truncated Stat protein makes up at least 30% of the total recombinant truncated Stat protein produced. In a more preferred embodiment the soluble truncated Stat protein makes up at least 50% of the total recombinant truncated Stat protein produced. In one embodiment, the truncated Stat protein has an amino acid 15 sequence substantially similar to SEQ ID NO:3. In another embodiment, the truncated Stat protein has an amino acid sequence of SEQ ID NO:3. In preferred embodiments, the truncated Stat protein is purified. In one variation of this type, the purified truncated Stat protein exhibits a single protein band on 7% SDS-PAGE, run under reducing conditions.
- 20 The Stat proteins, including the truncated Stat proteins of the present invention are activated when a tyrosine residue of the protein is phosphorylated. In a preferred embodiment of this type, the phosphorylated tyrosine is tyrosine 701 of the Stat1 $\alpha$  amino acid sequence shown in SEQ ID NO:1.
- 25 In one embodiment, the purified truncated Stat protein is substantially or completely free of its phosphorylated form. In another embodiment, the purified truncated Stat protein is substantially or completely phosphorylated. In yet a third embodiment, the purified truncated Stat protein is a mixture of the nonphosphorylated and phosphorylated forms.
- 30 One embodiment of the present invention is a purified Stat protein that is either substantially or completely free of its corresponding phosphorylated, activated form or in the alternative, is essentially or entirely in the corresponding phosphorylated, activated form. One variation of this embodiment exhibits a single protein band on 7% SDS-PAGE, run under reducing conditions, and has an amino acid sequence substantially

- similar to SEQ ID NO:1. In another variation the purified Stat protein, exhibits a single protein band on 7% SDS-PAGE, run under reducing conditions, and has an amino acid sequence substantially similar to SEQ ID NO:2. Yet another variation also includes a purified Stat protein that exhibits a single protein band on 7% SDS-PAGE, run under
- 5 reducing conditions and has an amino acid sequence of SEQ ID NO:1. In still another variation of this embodiment, the purified Stat protein exhibits a single protein band on 7% SDS-PAGE, run under reducing conditions, and has an amino acid sequence of SEQ ID NO:2.
- 10 The truncated Stat proteins and purified Stat proteins including the purified truncated Stat proteins of the present invention can have a converted cysteine. The converted cysteine can be of the form of a modified cysteine, such as a cysteine having a blocked thiol group or of an analogue of cysteine such as homocysteine; or of an amino acid replacement for cysteine. In preferred embodiments of this last type, the amino acid replacement for
- 15 cysteine is an alternative polar neutral amino acid such as glycine, serine, threonine, tyrosine, asparagine, or glutamine. In more preferred embodiments of this type, the alternative polar neutral amino acid is a glycine, a serine, or a threonine. In preferred embodiments containing modified cysteines, the modified cysteine is as an alkylated cysteine, or a cysteine containing a mercurial, or the thiol is oxidized and forms a
- 20 disulfide bond with a second thiol moiety.

The alkylated cysteines may be alkylated by a variety of alkylating agents including iodoacetate, sodium tetracyanate, 5,5-dithiobis(2-nitrobenzoic acid), 2,2'-dithiobis-(5-nitropyridine) and N-ethyl maleimide (NEM). In preferred embodiments the alkylated

25 cysteines are alkylated by N-ethyl maleimide.

The purified truncated Stat proteins and purified Stat proteins, including the purified truncated Stat proteins of the present invention, can also have more than one converted cysteine. In one embodiment of this type, the Stat protein is Stat1 $\alpha$  or a fragment thereof

30 and has three converted cysteines at Cysteine 155, Cysteine 440, and Cysteine 492 of the Stat1 $\alpha$  amino acid sequence shown in SEQ ID NO:1. The three converted cysteines can take any form as listed above, including each cysteine taking an alternative form. In one such embodiment Cysteine 155 is alkylated, Cysteine 440 is substituted by homocysteine, and Cysteine 492 is substituted by a threonine. In a preferred embodiment, all three

converted cysteines are alkylated cysteines. All of these Stat proteins and purified Stat proteins can be purified to exhibit one band on 7% SDS-PAGE, under reducing conditions in either their phosphorylated, activated state or in their corresponding nonphosphorylated form.

5

The present invention also includes purified Stat N-terminal peptide fragments. These peptide fragments consist of a protein domain that can be selectively cleaved by mild proteolysis with subtilisin or proteinase K. The N-terminal peptide fragments can form homodimers. As part of a Stat protein, the N-terminal domain serves to enhance the 10 binding of two adjacent Stat dimers to a pair of closely aligned DNA binding sites, *i.e.*, binding sites separated by approximately 10 to 15 base pairs. In a preferred embodiment, the N-terminal peptide fragment has an amino acid sequence substantially similar to that of SEQ ID NO:4. In a more preferred embodiment, the N-terminal peptide fragment has an amino acid sequence of SEQ ID NO:4.

15

The present invention, also includes antibodies to the truncated Stat protein, and the N-terminal peptide fragment of a Stat protein, as purified from recombinant sources or produced by chemical synthesis, and derivatives or analogs thereof, including fusion proteins. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, 20 single chain, Fab fragments, and a Fab expression library. These antibodies may be labeled.

The present invention also includes nucleic acids comprising nucleotide sequences that encode a truncated Stat protein. In one embodiment the nucleic acid comprises a 25 nucleotide sequence that encodes a truncated Stat protein having an amino acid sequence that is substantially similar to SEQ ID NO:3. In a related embodiment the nucleic acid comprises a nucleotide sequence that encodes a truncated Stat protein having the amino acid sequence of SEQ ID NO:3. In yet another embodiment the nucleic acid comprises a nucleotide sequence that is substantially similar to SEQ ID NO:5 and codes for the 30 expression of a truncated Stat protein. In still another embodiment the nucleic acid contains a nucleotide sequence having the sequence of SEQ ID NO:5.

The present invention also includes nucleic acids that comprise a nucleotide sequence encoding an N-terminal fragment of a Stat protein. In one embodiment the nucleic acid

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comprises a nucleotide sequence that encodes a Stat N-terminal fragment having an amino acid sequence that is substantially similar to SEQ ID NO:4. In a related embodiment the nucleic acid comprises a nucleotide sequence that encodes a Stat N-terminal fragment having the amino acid sequence of SEQ ID NO:4. In yet another embodiment the nucleic  
5 acid comprises a nucleotide sequence that is substantially similar to SEQ ID NO:6 and codes for the expression of a Stat N-terminal fragment. In still another embodiment the nucleic acid contains a nucleotide sequence having the sequence of SEQ ID NO:6.

All of the nucleic acids of the present invention can also contain heterologous nucleotide  
10 sequences.

Methods of phosphorylating the Stat proteins *in vitro*, are also included in the present invention. In one embodiment the phosphorylation is performed with a preparation of EGF-receptor kinase. In preferred embodiments the EGF-receptor preparation is obtained  
15 from cell lysates and purified with the use of an anti-EGF-receptor antibody directed against the extracellular domain. In some such embodiments the resulting EGF-receptor antibody complex is precipitated with Protein A agarose beads. In another preferred embodiment the antibody is a monoclonal antibody. In yet another preferred embodiment the cell lysates are from humans. In the most preferred embodiment of this method, the  
20 antibody is a monoclonal antibody and the cell lysates are from humans.

The present invention also includes methods of separating phosphorylated Stat proteins including phosphorylated truncated Stat proteins from their nonphosphorylated counterparts. Although these methods may be properly applied to all Stat proteins, and  
25 their corresponding truncated proteins, in preferred embodiments the Stat protein has an amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2, and the truncated Stat protein has an amino acid sequence substantially similar to SEQ ID NO:3. In more preferred embodiments the Stat protein or the truncated Stat protein also has a converted cysteine. In the most preferred embodiment, the Stat protein or truncated Stat protein has three  
30 converted cysteines which are alkylated cysteines at Cysteine 155, Cysteine 440, and Cysteine 492 of the Stat1 $\alpha$  amino acid sequence shown in SEQ ID NO:1.

In one embodiment a mixture containing phosphorylated Stat protein and nonphosphorylated Stat protein are placed onto a heparin-solid support. In preferred

embodiments the heparin solid support is either heparin agarose, heparin SEPHADEX or heparin cellulose. In the most preferred embodiment the heparin-solid support is heparin agarose.

- 5 In one variation of this embodiment the heparin agarose is washed first with a low-salt buffer to remove materials that either bind more weakly than the nonphosphorylated Stat protein or do not bind at all. The Stat proteins are eluted from the heparin agarose as a function of salt concentration with the nonphosphorylated Stat protein eluting at a lower salt concentration than the phosphorylated protein. In one particular embodiment of this  
10 type, the protein is eluted with a salt gradient. In a preferred embodiment, the elution of the heparin agarose is performed stepwise with an approximately 0.15 M monovalent salt elution step, followed by an approximately 0.4 M monovalent salt elution step. In this case the unphosphorylated Stat protein elutes during the first elution step, and the phosphorylated Stat protein elutes during the second elution step. In a more preferred  
15 embodiment the monovalent salt is potassium chloride.

This procedure may be performed by a batchwise method, though in preferred embodiments the heparin agarose is placed in a column. The procedure may be performed by simple controlled pumping of the column, or by HPLC, FPLC and any  
20 other analogous methodology; or the column may be allowed to flow by the pressure of gravity.

The present invention also includes methods of preparing a purified alkylated Stat protein and methods of preparing a purified alkylated truncated Stat protein. Although these  
25 methods may be properly applied to all Stat and truncated Stat proteins, in preferred embodiments the Stat protein has an amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2, and the truncated Stat protein has an amino acid sequence substantially similar to SEQ ID NO:3. In one such embodiment an expression vector containing a nucleic acid that encodes a Stat protein is placed into a compatible host cell, and the Stat protein is  
30 expressed. The compatible host cell is grown, harvested and then the expressed Stat protein is released from the host cell. In a preferred embodiment the expressed Stat protein is released from the host cell by lysing the cells. The Stat protein is then treated with an alkylating agent to alkylate one or more cysteines involved in intersubunit aggregation. The alkylated Stat protein is then isolated, yielding a purified alkylated Stat

protein.

In another such embodiment, the expression vector contains a nucleic acid that encodes a truncated Stat protein. The truncated Stat protein has an amino acid sequence having an  
5 N-terminal sequence that is substantially similar to the N-terminus of the corresponding resulting Stat protein following the cleavage of the proteolytic sensitive N-terminal domain from the corresponding Stat protein. The carboxyl terminus of the truncated Stat protein extends at least to the phosphorylatable tyrosine required for homodimerization. In preferred embodiments, alkylation is performed by incubating the Stat protein with  
10 N-ethyl maleimide. In more preferred embodiments, about 40 to 50 mg of purified alkylated truncated Stat protein can be obtained from 6 liters of starting culture. These methods can also include a step of phosphorylating the Stat protein either prior to or preferably following alkylation. In preferred methods of this type, preparations of EGF-receptor kinase are used in the *in vitro* phosphorylating step.

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The present invention also includes methods of preparing a purified substituted Stat protein including methods of preparing a purified substituted truncated Stat protein. Although these methods may be properly applied to all Stat proteins including truncated Stat proteins, in preferred embodiments the Stat protein has an amino acid sequence of SEQ ID  
20 NO:1 or SEQ ID NO:2, and the truncated Stat protein has an amino acid sequence substantially similar to SEQ ID NO:3. In one such embodiment, an expression vector contains a nucleic acid that encodes a substituted Stat protein that has an alternative amino acid substituted for a cysteine of the Stat protein, thereby replacing it. In one preferred embodiment, the amino acid is a polar neutral amino acid. In a variation of this  
25 embodiment the alternative polar neutral amino acid is a glycine. In another variation of this embodiment, the alternative polar neutral amino acid is a serine. In still another variation of this embodiment, the alternative polar neutral amino acid is a threonine. In preferred embodiments, the cysteine that has been replaced was involved in the intersubunit aggregation that takes place between Stat proteins.

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The expression vector is then placed into a compatible host cell, and the substituted Stat protein is expressed. The compatible host cell is grown, harvested and then the expressed substituted Stat protein is released from the host cell. In a preferred embodiment the expressed Stat protein is released from the host cell by lysing the cells. The substituted

Stat protein is then isolated, yielding a purified substituted Stat protein.

In one embodiment, the expression vector contains a nucleic acid that encodes a substituted truncated Stat protein. In one such embodiment, an expression vector contains  
5 a nucleic acid that encodes a substituted truncated Stat protein that has an alternative polar neutral amino acid substituted for a cysteine of the Stat protein, thereby replacing it. In one variation of this embodiment, the alternative polar neutral amino acid is a glycine. In another variation of this embodiment, the alternative polar neutral amino acid is a serine. In yet another variation of this embodiment, the alternative polar neutral amino acid is a  
10 threonine. In a preferred embodiment, the cysteine that has been replaced was involved in the intersubunit aggregation that takes place between Stat proteins. The substituted truncated Stat protein has an amino acid sequence which is essentially the same as the protease-resistant domain of the Stat protein. In preferred embodiments, about 40 to 50 mg of purified substituted truncated Stat protein can be obtained from 6 liters of starting  
15 culture. These methods can also include a step of phosphorylating the Stat protein or truncated Stat protein. In a preferred methods of this type, an EGF-receptor kinase preparation is used in the *in vitro* phosphorylating step.

In some embodiments, a substituted Stat protein or a substituted truncated Stat protein is  
20 also alkylated. In such cases an expression vector containing a nucleic acid that encodes a substituted Stat protein or a substituted truncated Stat protein is placed into a compatible host cell, and expressed. In one embodiment the substituted Stat protein contains a replacement amino acid that is an alternative polar neutral amino acid. In a preferred embodiment the alternative polar neutral amino acid is a glycine, a serine, or a threonine.  
25 The compatible host cell is grown, harvested and then the expressed substituted Stat protein or substituted truncated Stat protein is released from the host cell as described herein. The substituted Stat protein or substituted truncated Stat protein is then treated with an alkylating agent to alkylate one or more cysteines involved in intersubunit aggregation. The alkylated substituted Stat protein or alkylated substituted truncated Stat  
30 protein is then isolated, yielding a purified alkylated substituted Stat protein or purified alkylated substituted truncated Stat protein. In preferred embodiments, alkylation is performed by incubating the Stat protein or truncated Stat protein with N-ethyl maleimide. In more preferred embodiments about 40 to 50 mg of purified alkylated substituted truncated Stat protein can be obtained from 6 liters of starting culture.

The present invention also includes methods of identifying drugs that effect the interaction of N-terminal domains of Stat proteins that are bound to adjacent DNA binding sites. In one such embodiment, a drug library is screened by assaying the binding activity of a Stat protein to its DNA binding site. This assay is based on the ability of the N-terminal domain of Stat proteins to substantially enhance the binding affinity of two adjacent Stat dimers to a pair of closely aligned DNA binding sites, *i.e.*, binding sites separated by approximately 10 to 15 base pairs. Such drug libraries include phage libraries as described below, chemical libraries compiled by the major drug manufacturers, mixed libraries, and the like. Any of such compounds contained in the drug libraries are suitable for testing as a prospective drug in the assays described below, and further in a high throughput assay based on the methods described below.

One such embodiment includes a method of identifying a drug that interferes with the interaction of the N-terminal domains of Stat proteins bound to DNA binding sites. One variation of this embodiment relies on a truncated Stat protein that is missing the N-terminal domain responsible for enhancing the binding of two adjacent Stat dimers to a pair of closely aligned DNA binding sites. The binding affinity of a Stat protein to a DNA binding site effected by the N-terminal interaction of Stat proteins is determined. The effect of a prospective drug on the affinity of the Stat protein-DNA binding is determined. If the prospective drug decreases the binding affinity of the Stat protein to a DNA binding site, it becomes a candidate drug. The binding affinity of the corresponding truncated Stat protein to that DNA binding site is also determined. The effect of a candidate drug on the affinity of the truncated Stat protein-DNA binding is determined. If the candidate drug has no effect on the truncated Stat protein-DNA binding, then it can be concluded that the candidate drug interferes with the interaction of N-terminal domains of Stat proteins bound to adjacent DNA binding sites. In a preferred embodiment, the truncated Stat protein has an amino acid sequence that is substantially similar to SEQ ID NO:3.

This variation also includes a method of identifying a drug that enhances the interaction of the N-terminal domains of Stat proteins bound to DNA binding sites. The binding affinity of a Stat protein to a DNA binding site effected by the N-terminal interaction of Stat proteins is determined. The effect of a prospective drug on the affinity of the Stat protein-DNA binding is determined. If the prospective drug increases the binding affinity of the

- Stat protein to a DNA binding site, it becomes a candidate drug. The binding affinity of the corresponding truncated Stat protein to that DNA binding site is also determined. The effect of a candidate drug on the affinity of the truncated Stat protein-DNA binding is determined. If the candidate drug has no effect on the truncated Stat protein-DNA
- 5 binding, then it can be concluded that the candidate drug enhances the interaction of N-terminal domains of Stat proteins bound to adjacent DNA binding sites. In a preferred embodiment, the truncated Stat protein has an amino acid sequence that is substantially similar to SEQ ID NO:3.
- 10 In another embodiment, a drug library is screened by assaying the binding activity of the two N-terminal fragments of the present invention. As disclosed in the present invention, the N-terminal fragments of Stat proteins form stable dimers in solution. These dimers could mimic the role the N-terminal domain plays in the native Stat protein. Therefore, a prospective drug capable of disrupting or enhancing the stability of the dimer formed
- 15 between two N-terminal fragments becomes a candidate for a drug capable of destabilizing or stabilizing respectively, N-terminal domain-dependent Stat-DNA binding. These candidate drugs then can be tested in an *in vitro* or *in vivo* assay with Stat proteins. For example, dimerization of the N-terminal fragments in solution can be determined using techniques such as fluorescence depolarization.
- 20 In yet another embodiment, an N-terminal fragment of a Stat protein is attached to a solid support. The solid support is washed to remove unreacted species. A solution of free N-terminal fragments is poured onto the solid support and the N-terminal fragments are allowed to form dimers with their bound counterparts. In one variation, the solid support
- 25 is washed again to remove N-terminal fragments that do not bind. Prospective drugs can be screened for their ability to disrupt the dimers, or the formation of the dimers, and thereby increase the concentration of free N-terminal fragments. In a variation of this embodiment, prospective drugs may be screened that enhance the binding of the free N-terminal fragments with their bound counterparts. In this case, there is a corresponding
- 30 decrease in the concentration free N-terminal fragments. In either case, the measurement of an equilibrium constant, or a dissociation rate constant or an off-rate, may be used to express the effect of the prospective drug on the N-terminal fragment dimer binding. In another variation of this embodiment, prospective drugs that modulate the interaction of the N-terminal domain can be screened by determining the amount of N-terminal fragment

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that remains bound in the presence of the prospective drug. As compared to the amount of bound fragment in the absence of a prospective drug, prospective drugs that disrupt the interaction result in lower levels of bound fragments, whereas prospective drugs which enhance the interaction result in higher levels of bound fragment. One method of monitoring such interactions is through the use of free N-terminal fragments which have been labeled. Some suitable labels are exemplified below. Alternatively, the dimerization of the free N-terminal fragments with the bound N-terminal fragments can be monitored by changes in surface plasmon resonance. In preferred embodiments the N-terminal fragment has an amino acid sequence substantially similar to SEQ ID NO:4.

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In yet another embodiment, the affect of a prospective drug (a test compound) on interactions between N-terminal domains of STATs is assayed in living cells that contain or can be induced to contain activated STAT proteins, *i.e.*, STAT protein dimers. Cells containing a reporter gene, such as the heterologous gene for luciferase, green fluorescent protein, chloramphenicol acetyl transferase or  $\beta$ -galactosidase, operably linked to a promoter comprising two weak STAT binding sites are contacted with a prospective drug in the presence of a cytokine which activates the STAT(s) of interest. The amount (and/or activity) of reporter produced in the absence and presence of prospective drug is determined and compared. Prospective drugs which reduce the amount (and/or activity) of reporter produced are candidate antagonists of the N-terminal interaction, whereas prospective drugs which increase the amount (and/or activity) of reporter produced are candidate agonists. Cells containing a reporter gene operably linked to a promoter comprising strong STAT binding sites are then contacted with these candidate drugs, in the presence of a cytokine which activates the STAT(s) of interest. The amount (and/or activity) of reporter produced in the presence and absence of candidate drugs is determined and compared. Drugs which disrupt interactions between the N-terminal domains of the STATs will not reduce reporter activity in this second step. Similarly, candidate drugs which enhance interactions between N-terminal domains of STATs will not increase reporter activity in this second step.

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In an analogous embodiment, two reporter genes each operably under the control of one of the two types promoters described above can be comprised in a single host cell as long as the expression of the two reporter gene products can be distinguished. For example, different modified forms of green fluorescent protein can be used as described in U.S.

Patent 5,625,048, Issued April 29, 1997, hereby incorporated by reference in its entirety.

Antagonists of the STAT N-terminal interaction would be expected to antagonize aspects of STAT function. Such candidate drugs are expected to be useful for the treatment of a variety of disease states, including but not limited to, inflammation, allergy, asthma, and leukemias. Candidate drugs which stabilize the N-terminal interaction would be expected to enhance STAT function, and may therefore have utility in the treatment of anemias, neutropenias, thrombocytopenia, cancer, obesity, viral diseases and growth retardation, or other diseases characterized by a insufficient STAT activity.

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These and other aspects of the present invention will be better appreciated by reference to the following drawings and Detailed Description.

#### BRIEF DESCRIPTION OF THE DRAWINGS

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Figure 1A. Polyacrylamide gel electrophoretic analysis of the purified nonphosphorylated proteins. Aliquots of Stat1 $\alpha$  (lane 2; 2 $\mu$ g), Stat1 $\beta$  (lane 3; 2 $\mu$ g), and Stat1tc (lane 4; 4 $\mu$ g) were run on a 7% SDS-PAGE gel and stained with Coomassie blue. Molecular weight standards were run in lane 1. M<sub>r</sub> is given as the kDa on the left.

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Figure 1B. Proteolysis of human Stat1 $\alpha$ . 40 $\mu$ g of purified Stat1 $\alpha$  were digested with various amounts of subtilisin (lanes 4-6) or proteinase K (lanes 9-11) for 30 min on ice (as described in Materials and Methods, *infra*). The ratios (wt/wt) of protease to protein were 1:8 (lanes 4 and 9), 1:80 (lanes 5 and 10), and 1:800 (lanes 6 and 11). Aliquots of the reactions were resolved on a 16.5% SDS-polyacrylamide gel followed by Coomassie staining. Lane 1, molecular weight standards in kDa; lanes 2 and 7, untreated Stat1 $\alpha$ ; lane 3, subtilisin (15 $\mu$ g); lane 8, proteinase K (15 $\mu$ g). Stable fragments of 65 kDa and 16 kDa (see text) are marked with arrows.

30 Figure 2A. Phosphorylation of Stat1 $\alpha$  with EGF-receptor kinase *in vitro*. 2 $\mu$ g of Stat protein was incubated with EGF-receptor and 1 $\mu$ Ci of  $^{32}$ P  $\gamma$ ATP for 6 h at 4°C. The reaction (20 $\mu$ l volume) was stopped by the addition of SDS-sample buffer, resolved on a 7% SDS-PAGE, which was subsequently dried and exposed to an X-ray film. The typical doublet pattern for phosphorylated Stat1 (Shuai *et al.*, 1992) is seen in the Coomassie

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stained gel in lane 2. Lane 3 shows the corresponding autoradiogram. Only the slower migrating band contains  $^{32}\text{P}$ . (\*) denotes the position of the phosphorylated EGF-receptor. Fast and slow migrating Stat proteins are pointed out with lines. Lane 1 contains the molecular weight markers and their respective molecular weights are denoted in kDa.

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- Figure 2B. Isolation of *in vitro* phosphorylated Stat1tc. A total of 25 mg of protein was loaded on a heparin agarose column after an *in vitro* phosphorylation reaction and removal of EGF-receptor (see Materials and Methods). Depicted is the column profile of UV-absorptive material eluted with successive steps of 50mM KCl, 150mM KCl, and 400mM 10 KCl. Five microliters of the indicated fractions (2.5 ml) were resolved by 7% SDS PAGE and stained with Coomassie blue (lower insert) or blotted on a nitrocellulose membrane and probed with an anti-phosphotyrosine-antibody (1:1500 diluted PY 20 (UBI); upper insert). Molecular weights are denoted in kDa.
- 15 Figure 2C. Tyrosine 701 is phosphorylated by EGF-receptor. The endoproteinase AspN digests (15 min) were carried out on alkylated Stat1 $\beta$  in either the unphosphorylated form (- phosph, upper half) or the chromatographically purified phosphorylated form (+ phosph, lower half). The relevant proteins of the matrix-assisted laser desorption/ionization mass spectrum are shown. Accurate molecular mass determinations 20 allowed for unequivocal identification of the peptide fragments. Peaks are labeled according to the amino acid sequence of the corresponding peptides.

- Figure 3A. DNA binding of purified phosphorylated Stat1 $\alpha$  (lane 1) and Stat1tc (lane 2) using as a probe the radioactively labelled cfosWT sequence. Binding reactions contained 25 equimolar amounts of the respective proteins. The position of migration of the free DNA probe (free) and the protein/DNA complex (bound) is indicated. Note the presence of a slower migrating band only with the full length Stat1 $\alpha$ , lane 1 (see also Figure 3B).

- Figure 3B. Influence of cysteine alkylation on the DNA binding activity of Stat1 $\alpha$ . A 30 mixture of phosphorylated and unphosphorylated protein (0.23  $\mu\text{M}$  final; ~ 15% phosphoprotein) was reacted in the presence of 0.8 mM DTT and the indicated concentrations of N-ethyl-maleimide (NEM) for 20 min at room temperature in a volume of 12.5  $\mu\text{l}$ . The reaction was stopped with DTT (final concentration of 10 mM) followed by the addition of 1.5 pmoles of labelled probe (cfosM67). Samples were resolved on a

4.5% native polyacrylamide gel. (M) denotes the position of bromophenol blue (lower) and xylene cyanol (upper) markers.

Figure 4. Titration of  $^{32}\text{P}$  labelled cfosWT oligonucleotide with phosphorylated Stat1tc  
 5 and full length Stat1 $\alpha$ . A fixed amount of  $^{32}\text{P}$  labelled cfosWT oligonucleotide ( $5.6 \times 10^{-10}$   
 M) was incubated with Stat1 proteins in a  $12.5 \mu\text{l}$  volume as described in Materials and  
 Methods. Numbers above the lanes indicate the concentrations of dimeric Stat1 $\alpha$  and  
 Stat1tc in each reaction. Protein-bound (bound) and free (free) DNA is identified. The  
 concentration of free protein dimers at half saturation was determined to be approximately  
 10  $1 \text{nM}$  in both cases which corresponds to the apparent equilibrium constant  $K_{eq}$ . In the  
 lanes marked above "DNA only" no Stat protein was included in the reaction.

Figure 5A. Titration of phosphorylated truncated Stat1 protein with  $^{32}\text{P}$  labelled  
 oligonucleotides containing a "low" (Ly6\_E, left panel) or "high" (S1, right panel)  
 15 affinity binding site. The DNA concentration was fixed at  $2.6 \times 10^{-10} \text{ M}$  and titrated in a  
 $12.5 \mu\text{l}$  volume against a standard protein dilution series ranging from  $5 \times 10^{-11} \text{ M}$  to  $2.6 \times 10^{-8} \text{ M}$  dimer final. Protein concentrations for the dimeric protein are given above each  
 lane. The products were resolved on a native 4.5% polyacrylamide gel and quantified as  
 described in experimental procedures. (Bound) protein/DNA complex; (free) free DNA.  
 20 There was no Stat1tc included in reactions run on lanes denoted "only DNA". The dimer  
 concentration at half saturation was determined from this autoradiograph to be  
 approximately  $1 \times 10^{-9} \text{ M}$  for both DNA sequences.

Figure 5B. The complex of Stat1 $\alpha$  with cfosWT DNA is less stable than the complex  
 25 with cfosM67 DNA. Results are shown for experiments designed to determine the off-rate  
 in which  $0.55 \times 10^{-9} \text{ M}$  dimer was prebound with the radiolabelled DNA fragments (at  $2 \times 10^{-9} \text{ M}$ ) containing the cfosWT (0 min; left panel) or cfosM67 (0 min; right panel)  
 sequences. Excess unlabelled DNA (100 x molar excess) was added to the reaction at time  
 zero, and aliquots were taken at the indicated intervals and loaded onto a running gel to  
 30 visualize the amount of complex remaining. The half life of the Stat1 $\alpha$ /cfosWT complex is  
 less than 0.5 min and that for the Stat1tc/cfosM67 complex in this titration is about 3 min.  
 Because the electrophoresis was continuous during the experiment the DNA fragments  
 (free) and the complexes (bound) are located progressively higher on the gel with  
 increasing time, because the later samples were electrophoresed for shorter periods of time

than were the earlier ones.

Figure 6A. Comparison of the dissociation rates of complexes containing DNA fragments with two consecutive binding sites (2x *cfos*WT, 10bp apart) and Stat1 $\alpha$  (right) or Stat1tc (left). 0.5 x 10<sup>-9</sup> M dimer was prebound with 0.7 x 10<sup>-9</sup> M radiolabelled DNA for 5 min at room temperature (lanes 1 and 8). After the addition of a 100-fold molar excess of unlabelled DNA at time point zero the reaction was further incubated for the times indicated before aliquots were loaded on a running polyacrylamide gel. At time zero two differently migrating complexes are visible, denoted "(2 x (Dimer))" and "Dimer".

10 Unbound (free) DNA runs at the bottom of the gel.

Figure 6B. Identification of the amino terminal 131 amino acids as functional in (2 x (Dimer)) stabilization on DNA. Comparison of stability of Stat1 $\beta$  (lanes 5 - 8) and Stat1tc (lanes 1 - 4) on DNA fragments containing two consecutive binding sites (2x *cfos*WT, 15 10bp apart). The experimental protocol was the same as in Figure 6A.

Figure 7A. Influence of promotor orientation on protein/DNA complex formation and stability. 1.65 x 10<sup>-9</sup> M Stat1 $\alpha$  dimer were equilibrated with labelled DNA (0.7 x 10<sup>-9</sup> M) with two consecutive binding sites (2x *cfos*WT) 10bp apart in parallel (lanes 1- 4) or 20 antiparallel (lanes 5 - 8) orientation. The preformed complexes were chased with unlabelled competitor DNA as described in the legend to Figure 6A.

Figure 7B. Stat1 $\alpha$  binding to DNA fragments with two parallel binding sites (2x *cfos*WT) spaced 10bp (lanes 1-4), 5bp (lanes 5-8), or 15bp (lanes 9-12). The chase experiment was 25 performed as described in the legend to Figure 6A.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention includes methods for producing milligram quantities of different 30 forms of purified Stat proteins from recombinant DNA constructs. One key aspect of the present invention is the isolation of purified phosphorylated Stat proteins. Another key aspect of the methods of the present invention comprises the modification of specific cysteine residues of the Stat proteins that prevent aggregation. In one preferred embodiment, the modification of the cysteine residues is performed by alkylation.

The present invention also includes a stable, soluble truncated Stat protein that retains most of the functional activities of the corresponding native Stat protein. Since a significant portion of the recombinant truncated Stat protein does not form inclusion bodies and therefore can be isolated in large quantities (40-50 mg of purified alkylated 5 truncated Stat1 protein can be obtained from 6 liters of starting culture,) it is an excellent source of protein for the critical *in vitro* studies necessary to understand and later, control the signal transducing properties of Stat proteins. Nucleic acids that encode for a truncated Stat protein are also a part of the present invention. The present invention also includes methods of using the truncated Stat proteins for identifying drugs that specifically 10 effect the interaction of N-terminal domains of Stat proteins that are bound to adjacent DNA binding sites.

The present invention includes the identification and isolation of an N-terminal fragment comprised of a compact domain in the amino terminus of Stat1 $\alpha$ . This compact domain 15 enhances the DNA binding of the Stat protein due to its ability to interact with a neighboring Stat protein. Methods of using this N-terminal fragment to identify specific drugs that act to either prevent or enhance the DNA binding of Stat proteins through interfering with or promoting the inter-protein interaction of the N-terminal domain of Stat proteins are also included.

20 The present invention also includes methods of phosphorylating, *in vitro*, the tyrosine residue of Stat proteins, known *in vivo* to cause the dimerization of the Stat protein upon being phosphorylated. In one preferred embodiment, activated EGF-receptor partially purified from membranes by immunoprecipitation is used to catalyze this phosphorylation.

25 In addition, the present invention includes methods of separating a phosphorylated Stat protein from its corresponding nonphosphorylated form. Heretofore, such separation could not be achieved due to the unusual behavior of Stat proteins on gel filtration columns.

30 Therefore, if appearing herein, the following terms shall have the definitions set out below.

As used herein a "converted cysteine" implies that a cysteine residue of a Stat protein or

truncated Stat protein of the present invention has either been modified or replaced by an alternative naturally occurring or synthetic amino acid. A converted cysteine can be of the form of a modified cysteine such as a cysteine having its thiol group blocked, an analogue of cysteine such as homocysteine, or an amino acid replacement for cysteine  
5 such as a glycine or a serine. Modification of the modified cysteines can be accomplished through alkylation (*i.e.*, forming an alkylated cysteine), or by mercuration, or through disulfide bond formation.

As used herein a the term "Stat protein" includes a particular family of transcription factor  
10 consisting of the Signal Transducers and Activators of Transcription proteins. These proteins have been defined in International Patent Publication No.s WO 93/19179 (30 September 1993, by James E. Darnell, Jr. *et al.*), WO 95/08629 (30 March 1995, by James E. Darnell, Jr. *et al.*) and United States application having a Serial Number 08/212,184, filed on March 11, 1994, entitled, "Interferon Associated Receptor  
15 Recognition Factors, Nucleic Acids Encoding the Same and Methods of Use Thereof" by James E. Darnell, Jr. *et al.*, all of which are incorporated by reference in their entireties, herein. Currently, there are seven mammalian Stat family members which have been identified, numbered Stat 1, 2, 3, 4, 5A, 5B, and 6. Stat proteins include proteins derived from alternative splice sites such as Human Stat1 $\alpha$  and Stat1 $\beta$ , *i.e.*, Stat1 $\beta$  is a shorter  
20 protein than Stat1 $\alpha$  and is translated from an alternatively spliced mRNA. Modified Stat proteins and functional fragments of Stat proteins are included in the present invention. One functional fragment is a truncated Stat protein defined below.

As used herein a the term "truncated Stat protein" denotes a Stat protein fragment having  
25 an N-terminal amino acid sequence that is substantially similar to the N-terminus of the corresponding full-length Stat protein following the cleavage of the proteolytic sensitive N-terminal domain from the corresponding full-length Stat protein. The carboxyl terminus of the truncated Stat protein extends at least to the phosphorylatable tyrosine required for homodimerization. Truncated Stat proteins are soluble proteins that can be  
30 phosphorylated, dimerize and bind to the DNA binding sites of the full-length Stat protein. An example of a truncated Stat protein is Stat1tc having the amino acid sequence of SEQ ID NO:3.

As used herein the terms "phosphorylated" and "nonphosphorylated" as used in

conjunction with or in reference to a Stat protein denote the phosphorylation state of a particular tyrosine residue of the Stat proteins (e.g., Tyr 701 of Stat1). When Stat proteins are phosphorylated, they form homo- or heterodimeric structures in which the phosphotyrosine of one partner binds to the SRC homology domain (SH2) of the other. In 5 their natural environment the newly formed dimer then translocates from the cytoplasm to the nucleus, binds to a palindromic GAS sequence, thereby activating transcription

In a specific embodiment, two amino acid sequences of the truncated Stat protein are "substantially homologous" or "substantially similar" when at least about 75% (preferably 10 at least about 90%, and most preferably at least about 95 or 98%) of the amino acids match over the defined length of the amino acid sequences; and the N-terminal domain of the corresponding full-length Stat protein is at least fifty percent deleted from both amino acid sequences. Analogously, two amino acid sequences of the Stat N-terminal peptide fragments are "substantially homologous" or "substantially similar" when at least about 15 75% (preferably at least about 90%, and most preferably at least about 95 or 98%) of the amino acids match over the defined length of the amino acid sequences; and the N-terminal peptide fragment can form homodimers. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks.

20 In a specific embodiment, two nucleotide sequences coding for the expression of the truncated Stat protein of the present invention are "substantially homologous" or "substantially similar" when at least about 50% (preferably at least about 75%, and most preferably at least about 90 or 95%) of the nucleotides match over the defined length of 25 the nucleotide sequences; and the coding region for the N-terminal domain of the corresponding full-length Stat protein is at least fifty percent deleted (or frame-shifted from the coding region) from both nucleotide sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks.

30

Purification and Characterization of the Stat protein and the truncated Stat protein

The Stat protein and truncated Stat protein of the present invention and homologues thereof can be purified as taught herein, using any number of alternative equivalent

procedures that encompass a wide variety of known purification steps. Those with skill in the art would know to refer to references, such as the Methods of Enzymology series, for greater detail and breadth.

- 5 In a specific embodiment, exemplified *below*, a suitable procedure for purifying a Stat protein of the present invention is described as follows. One skilled in the art of protein purification would know that any such general procedure would probably need to be modified for any given Stat protein and as such, performing the requisite modifications would not be considered undue experimentation.

10

*Expression and purification of a recombinant Stat protein.*

Nucleic acids containing sequences coding for a Stat protein are amplified by PCR with primers containing restriction sites in addition to homologous sequence. The products are then cloned using the restriction sites into a baculovirus transfer vector, *e.g.* pAcSG2.

- 15 Recombinant vectors are subsequently co-transfected with baculovirus DNA, such as Baculogold, into Sf9 insect cells. Recombinant viruses can be identified by immunoblot of extracts of the infected cells. For protein production Sf9 cells in a suspension culture (approximately 10<sup>6</sup> cells/ml) are infected with recombinant viruses (multiplicity of infection: 1:5) and harvested by low speed centrifugation approximately two days  
20 following infection.

The resulting cells, generally in quantities of between 10<sup>8</sup> - 10<sup>9</sup>, are lysed in ice cold extraction buffer [approximately 80 mls of a low concentration Mes buffer (20-50 mM) containing, 100 mM KCl, 10 mM NaF, 0.02% NaN<sub>3</sub>, 4 mM EDTA, 1 mM EGTA, 20 mM DTT, and Complete<sup>TM</sup> protease inhibitors (Boehringer Mannheim), pH adjusted with sodium hydroxide to pH 7.0] with a dounce homogenizer. All subsequent steps are performed at 4°C unless noted otherwise. For optimal results all buffers used during protein purification are chilled, thoroughly degassed and flushed with N<sub>2</sub> before use.

- 25  
30 The resulting lysates are cleared by low speed centrifugation. The supernatant is brought to about pH 6 after the addition of 0.5 vol of a buffer such as 20mM Mes containing 0.02% NaN<sub>3</sub>, 20 mM DTT, pH adjusted to about 6.0) and the supernatant is again centrifuged. The clarified supernatant is loaded onto a cation exchange resin, *e.g.*, S-SEPHAROSE, in a short, fat column, *e.g.*, 5 x 5.5 cm, and eluted with a linear salt

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- gradient (50-300 mM monovalent salt) and pH gradient (pH 6-7). Fractions containing Stat protein are identified by, *e.g.*, immunoblot, then pooled, and the pH of the pooled fractions are adjusted to 8.0 with 1M Tris. After the addition of 0.25 vol of a low concentration buffer such as 20 mM Tris-HCl containing 0.02% NaN<sub>3</sub>, 10 mM DTT, at 5 about pH 8, the solution is loaded onto an anionic exchange resin, such as Q-Sepharose, in a *e.g.*, a 2 x 9 cm column. The Stat protein is eluted with a linear monovalent salt gradient from 100 mM to 300 mM. Eluted Stat protein is precipitated with solid ammonium sulfate to 60% saturation. The resulting concentrated Stat proteins are dissolved in about 10 ml of 50 mM phosphate buffer, pH 7.2, containing 2 mM DTT, 10 mM EDTA, and Complete™ protease inhibitors. The Stat protein is then alkylated. In one embodiment, alkylation is performed with N-ethyl-maleimide which is added to a final concentration of 20 mM. The alkylation reaction mixture is incubated at room temperature for 10 min and then placed on ice for another 30 min. The reaction is stopped by the addition of β-mercaptoethanol to 50 mM and ammonium sulfate to 0.5 M. 15 The resulting reaction mixture is then loaded onto a low substituted Phenyl-Sepharose column (*e.g.*, 2 x 15 cm) equilibrated in a low concentration buffer such as 20 mM Tris-HCl, about pH 7.4, containing 2 mM DTT *plus* 0.5 M ammonium sulfate. The Stat proteins are eluted with decreasing ammonium sulfate dissolved in the column equilibration buffer. Fractions containing Stat protein are pooled, and then concentrated 20 to about 10 mg/ml using *e.g.*, a centriprep 50. The concentrated sample is then applied to a gel filtration column, such as SUPERDEX 200 (XK 16, Pharmacia) equilibrated in low concentration buffer such as 20 mM Hepes-HCl, pH 7.2, containing 0.02% NaN<sub>3</sub>, 2 mM DTT, and 0.3 M KCl. Fractions containing the Stat protein are pooled. The pooled fractions are then concentrated by ultrafiltration to approximately 20 mg/ml and quick 25 frozen on dry ice. The purified proteins are stored at -70°C. When purifying substituted Stat protein containing converted cysteines, in which the cysteines that are involved in the inter-protein aggregation have been replaced, the alkylation step is left out. The procedure is otherwise analogous.
- 30 *Expression and purification of a truncated Stat protein.*  
A portion of a Stat gene encoding a truncated Stat protein is amplified by PCR with primers containing restriction sites in addition to the desired sequence. The products are then cloned into a bacterial vector, *e.g.*, the pET20b expression vector (Novagen) using these restriction sites. Growth and induction of transformed E. coli *e.g.*, BL21DE3

- (pLysS) is performed by standard procedures, such as described by Studier and Moffatt, 1986 (in this particular case the induction was carried out for 4 hours at 30°C with 0.5 mM ITPG). Generally, about 50% of the induced protein remains soluble. This soluble truncated Stat protein is the isolatable form of the recombinant protein. Cells are
- 5 collected by centrifugation and resuspended in ice cold extraction buffer at a concentration of about 30 g of cells to 100 mls of a low concentration buffer, *e.g.*, 20 mM Hepes/HCl pH 7.6, containing 0.1 M KCl, 10% Glycerol, 1mM EDTA, 10 mM MnCl<sub>2</sub>, 20 mM DTT, 100 U/ml DNase I (Boehringer Mannheim), and Complete™ protease inhibitor. Cells are lysed by multiple cycles of freeze/thawing. Lysis is continued at 4°C while
- 10 stirring slowly for about an hour. The lysate is then centrifuged for about 20 min at about 20,000 x g at 4°C. Polyethylenimine (0.1% final; Sigma) is added to the supernatant, the solution gently mixed and centrifuged for about 15 min at about 15,000 x g. All subsequent steps are performed in the cold (4°C) unless stated otherwise.
- 15 The supernatant containing the soluble truncated Stat protein is precipitated with saturated ammonium sulfate solution in two steps (0-35%; 35-55% saturation final). The 35-55% pellet is redissolved in about 20 ml of 50 mM phosphate buffer, pH 7.2, containing 2 mM DTT, 1 mM EDTA, and Complete™ protease inhibitors. The truncated Stat protein is then alkylated. In one embodiment, alkylation is performed with N-ethyl-maleimide
- 20 which is added to a final concentration of 20 mM. The alkylation reaction mixture is incubated at room temperature for 10 min and then placed on ice for another 30 min. The reaction is stopped by the addition of  $\beta$ -mercaptoethanol to 50 mM and solid ammonium sulfate to 0.9 M. The mixture is then loaded onto a Fast Flow Phenyl-Sepharose column (low substituted, 2 x 15 cm) that had been equilibrated in buffer such as 50 mM Tris/HCl,
- 25 pH 7.4 containing 1 mM EDTA, 0.02% NaN<sub>3</sub>, 2 mM DTT, *plus* 0.9M ammonium sulfate. After washing the column, a linear decreasing salt gradient from 0.9 M to 0.05 M ammonium sulfate in the equilibration buffer, is applied. The truncated Stat protein elutes at about 0.5 M salt. The fractions containing truncated Stat protein are pooled and dialysed overnight against 2 x 4 liters of a buffer such as 40 mM Mes/NaOH pH 6.5,
- 30 containing 10% Glycerol, 0.5 mM EDTA, 0.02% NaN<sub>3</sub>, and 140 mM KCl. This material is loaded onto a cation exchange resin, *e.g.*, S-Sepharose, in a short, fat column, *e.g.*, 5 x 5.5 cm, and a linear 500 ml gradient of a buffer such as 40 mM Mes/NaOH pH 6.5, containing 10% Glycerol, 0.5 mM EDTA, 0.02% NaN<sub>3</sub> containing 140 mM to 300 mM KCl was applied. The protein generally elutes at approximately 220 mM KCl. Fractions

containing the truncated Stat protein are collected and dialysed against 3 liters of a buffer such as 50 mM Tris/HCl, pH 8 containing 10% Glycerol, 2mM DTT, and 50 mM KCl with one change of buffer. The protein solution is loaded onto an anionic exchange resin, such as Q-Sepharose, in a column *e.g.*, a 2 x 9 cm and bound proteins are eluted with a linear gradient from 50 to 300 mM KCl in a buffer such as 50 mM Tris/HCl, pH 8 containing 10% Glycerol, 2mM DTT. Fractions containing the truncated Stat protein are combined and precipitated with solid ammonium sulfate to 55% saturation. At this stage the 95% pure preparation can be stored at -20°C until subjected to *in vitro* phosphorylation or is directly loaded onto a gel filtration column, such as Superdex 200 (XK 16; Pharmacia) equilibrated with 10 mM Hepes/HCl, 7.4 containing 100 mM KCl, 2 mM DTT, and 0.5 mM EDTA. In this case the precipitated protein is first dissolved in about 2 ml of 10 mM Hepes/HCl, 7.4 containing 100 mM KCl, 2 mM DTT, and 0.5 mM EDTA and then placed on the gel filtration column. The truncated Stat protein elutes in a symmetrical peak and is concentrated to a concentration of about 20 mg/ml using a Centriprep 50, for example, and quick frozen on dry ice. The pure alkylated truncated Stat protein is stored at -70°C. Typically yields of 40-50 mg (greater than 98% pure as judged by Coomassie blue stain and mass spectroscopy) of truncated Stat protein from 6 liters of starting culture can be obtained. Any person skilled in the art would know to scale-up this procedure when a greater quantity of Stat protein is needed, and to scale-down the procedure when less purified Stat protein is required.

When purifying substituted truncated Stat protein containing converted cysteines, in which the cysteines that are involved in the inter-protein aggregation have been replaced, the alkylation step is left out. The procedure is otherwise analogous.

One key aspect of the present invention need to be emphasized: the identification of a soluble truncated Stat protein that is crucial for preparing large amounts (30-50 mgs) of Stat protein in a single preparation. Heretofore, essentially all of the recombinant Stat protein expressed in a bacterial host, accumulated entirely in insoluble inclusion bodies. The present invention has overcome this problem by producing a truncated protein that is soluble in significant quantities.

*Preparation of EGF-receptor kinase and in vitro phosphorylation of Stat proteins.* Human carcinoma cells such as A431 cells, are grown to 90% confluence in 150 mm diameter

plates in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum. The cells are washed once with chilled phosphate buffered saline, PBS, and lysates are then conveniently prepared in about 1 ml of ice cold lysis buffer per plate, such as 10mM Hepes/HCl, pH 7.5, containing 150 mM NaCl, 0.5% Triton X-100, 10% Glycerol, 1 mM 5  $\text{Na}_3\text{VO}_4$ , 10 mM EDTA and Complete<sup>TM</sup> protease inhibitors. After about 10 minutes on ice, the cells are scraped, vortexed and dounce homogenized. The lysates are cleared by centrifugation at 4°C, *e.g.*, by centrifuging for 20 min at top speed in an Eppendorf microfuge. The resulting supernatant is stored at -70°C until needed. Immediately before use, one volume of the lysates is mixed with four volumes of the lysis buffer forming 10 diluted lysate.

EGF-receptor precipitates are obtained by incubating 5 ml of diluted lysate with about 50  $\mu\text{g}$  of an anti-EGF-receptor monoclonal antibody directed against the extracellular domain. After two hours of rotating the sample at 4°C, 750  $\mu\text{l}$  of Protein-A-agarose (50% slurry; 15 Oncogene Science) is added, and the incubation proceeds while rotating, for about one more hour. Agarose beads containing the EGF-receptor immunoprecipitates are washed exhaustively (5-10 times) with lysis buffer and then at least twice more with a storage buffer such as 20 mM Hepes/HCl containing 20% Glycerol, 100 mM NaCl, and 0.1 mM  $\text{Na}_3\text{VO}_4$ . Precipitates from 5 ml diluted lysate are dissolved in 0.5 ml of the storage 20 buffer, flash frozen on dry ice and stored at -70°C.

Immediately before the in vitro kinase reaction the Protein-A-agarose bound EGF-receptor from 5 ml dilute lysate is washed once with a 1x kinase buffer such as, 20 mM Tris/HCl, pH 8.0 containing 50 mM KCl, 0.3 mM  $\text{Na}_3\text{VO}_4$ , 2 mM DTT, pH 8.0 and then dissolved 25 in 0.4 ml (total volume) of this buffer. Afterwards the washed EGF-receptor precipitate is incubated on ice for about 10 minutes in the presence of a final concentration of mouse EGF of 0.15 ng/ $\mu\text{l}$ . Phosphorylation reactions are conveniently carried out in Eppendorf tubes in a final volume of 1 ml. To the pre-incubated kinase preparation the following is 30 added: 60  $\mu\text{l}$  10x kinase buffer, 20  $\mu\text{l}$  0.1 M DTT, 50  $\mu\text{l}$  0.1 M ATP, 4 mg purified Stat protein (*e.g.*, the Superdex 200 eluate for Stat proteins; and ammonium sulfate pellets dissolved in 20 mM Tris /HCl, pH 8.0 for the truncated Stat protein of the preparations described above), 10  $\mu\text{l}$  1M MnCl<sub>2</sub> and distilled water is added to 1 ml. The reaction is allowed to proceed for about 15 hours at 4°C. After 3 hours an additional 15  $\mu\text{l}$  of 0.1 M ATP is added.

*Separation of phosphorylated from unphosphorylated Stat proteins.* The *in vitro* kinase reaction mixture (above) is freed from the EGF-receptor bound to agarose beads by washing the beads and physically separating the eluate from the beads. This may be conveniently performed by spinning the mixture through a plug of siliconized glass wool

- 5 at the bottom of a pierced Eppendorf tube. The glass wool is washed with 0.5 ml of a buffer, such as 20 mM Tris/HCl, pH 8.0, containing 1mM EDTA, and 2 mM DTT. This buffer is also used to equilibrate a heparin agarose column, (HA-buffer). The pooled volumes from the glass wool eluate are loaded onto the equilibrated heparin agarose column (1.5 x 7 cm) and the column is washed with about 50 ml HA-buffer *plus* 50 mM KCl. The bound Stat proteins or truncated Stat proteins are eluted with two consecutive 50 ml volumes of HA-buffer *plus* a moderate salt concentration such as 150 mM KCl and then HA-buffer plus a higher salt concentration such as 400 mM KCl. Unphosphorylated proteins generally elute at the moderate salt concentration and are then concentrated *e.g.*, by ultrafiltration to about 10 mg/ml, flash frozen on dry ice and stored at -70°C.

10 15 Phosphorylated Stat proteins generally elute at the higher salt concentration and are concentrated to about 1mg/ml. Glycerol is added to about 50% (vol/vol) and the material is stored at -20°C.

Phosphorylated truncated Stat protein is brought to a concentration of about 15 mg/ml.

- 20 The concentrated sample is then applied to a gel filtration column, such as Superdex 200 (XK 16, Pharmacia) equilibrated in low concentration buffer such as 20mM Hepes-HCl, pH 7.2, containing 0.02% NaN<sub>3</sub>, 2 mM DTT, and 0.3 M KCl. Fractions containing the gel filtered phosphorylated truncated Stat protein are pooled, concentrated to approximately 20 mg/ml, flash frozen on dry ice and stored at -70°C.

General Techniques for Constructing Nucleic Acids That Express Recombinant Stat Proteins

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook et al., 1989"); *DNA Cloning: A Practical Approach*, Volumes I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* [B.D. Hames

& S.J. Higgins eds. (1985)]; *Transcription And Translation* [B.D. Hames & S.J. Higgins, eds. (1984)]; *Animal Cell Culture* [R.I. Freshney, ed. (1986)]; *Immobilized Cells And Enzymes* [IRL Press, (1986)]; B. Perbal, *A Practical Guide To Molecular Cloning* (1984); F.M. Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994).

Therefore, if appearing herein, the following terms shall have the definitions set out below.

- 10 As used herein, the term "gene" refers to an assembly of nucleotides that encode a polypeptide, and includes cDNA and genomic DNA nucleic acids.

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment. A 15 "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*, *i.e.*, capable of replication under its own control.

- 20 A "cassette" refers to a segment of DNA that can be inserted into a vector at specific restriction sites. The segment of DNA encodes a polypeptide of interest, and the cassette and restriction sites are designed to ensure insertion of the cassette in the proper reading frame for transcription and translation.

A cell has been "transfected" by exogenous or heterologous DNA when such DNA has 25 been introduced inside the cell. A cell has been "transformed" by exogenous or heterologous DNA when the transfected DNA effects a phenotypic change. Preferably, the transforming DNA should be integrated (covalently linked) into chromosomal DNA making up the genome of the cell.

- 30 A "nucleic acid molecule" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester analogues thereof, such as phosphorothioates and thioesters, in either single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-

RNA and RNA-RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear or circular DNA molecules

5 (e.g., restriction fragments), plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a

10 molecular biological manipulation.

A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of

15 temperature and solution ionic strength (*see* Sambrook et al., *supra*). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. For preliminary screening for homologous nucleic acids, low stringency hybridization conditions, corresponding to a  $T_m$  of 55°, can be used, *e.g.*, 5x SSC, 0.1% SDS, 0.25% milk, and no formamide; or 30% formamide, 5x SSC, 0.5% SDS). Moderate stringency

20 hybridization conditions correspond to a higher  $T_m$ , *e.g.*, 40% formamide, with 5x or 6x SCC. High stringency hybridization conditions correspond to the highest  $T_m$ , *e.g.*, 50% formamide, 5x or 6x SCC. Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing

25 nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of  $T_m$  for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher  $T_m$ ) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA,

30 DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating  $T_m$  have been derived (*see* Sambrook et al., *supra*, 9.50-0.51). For hybridization with shorter nucleic acids, *i.e.*, oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (*see* Sambrook et al., *supra*, 11.7-11.8). Preferably a minimum length for a hybridizable

nucleic acid is at least about 12 nucleotides; preferably at least about 18 nucleotides; and more preferably the length is at least about 27 nucleotides; and most preferably 36 nucleotides.

- 5 In a specific embodiment, the term "standard hybridization conditions" refers to a  $T_m$  of 55°C, and utilizes conditions as set forth above. In a preferred embodiment, the  $T_m$  is 60°C; in a more preferred embodiment, the  $T_m$  is 65°C.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and  
10 translated into a polypeptide in a cell *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences and synthetic DNA sequences. If the coding sequence is intended for  
15 expression in a eukaryotic cell, a polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, terminators, and the like, that provide for the expression of a  
20 coding sequence in a host cell. In eukaryotic cells, polyadenylation signals are control sequences.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For  
25 purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as  
30 protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA,

which is then trans-*tRNA* spliced and translated into the protein encoded by the coding sequence.

A "signal sequence" is included at the beginning of the coding sequence of a protein to be expressed on the surface of a cell. This sequence encodes a signal peptide, N-terminal to the mature polypeptide, that directs the host cell to translocate the polypeptide. The term "translocation signal sequence" is used herein to refer to this sort of signal sequence.

Translocation signal sequences can be found associated with a variety of proteins native to eukaryotes and prokaryotes, and are often functional in both types of organisms.

10

As used herein, the term "homologous" in all its grammatical forms refers to the relationship between proteins that possess a "common evolutionary origin," including proteins from superfamilies (*e.g.*, the immunoglobulin superfamily) and homologous proteins from different species (*e.g.*, myosin light chain, etc.) (Reeck et al., 1987, Cell 50:667). Such proteins have sequence homology as reflected by their high degree of sequence similarity.

Accordingly, the term "sequence similarity" in all its grammatical forms refers to the degree of identity or correspondence between nucleic acid or amino acid sequences of proteins that may or may not share a common evolutionary origin (*see* Reeck et al., *supra*). However, in common usage and in the instant application, the term "homologous," when modified with an adverb such as "highly," may refer to sequence similarity and not a common evolutionary origin.

25 The term "corresponding to" is used herein to refer similar or homologous sequences, whether the exact position is identical or different from the molecule to which the similarity or homology is measured. Thus, the term "corresponding to" refers to the sequence similarity, and not the numbering of the amino acid residues or nucleotide bases.

30 A gene encoding Stat protein, whether genomic DNA or cDNA, can be isolated from any animal source, particularly from a mammal. Methods for obtaining the *Stat protein* gene are well known in the art, as described above (see, e.g., Sambrook et al., 1989, *supra*).

A "heterologous nucleotide sequence" as used herein is a nucleotide sequence that is added

to a nucleotide sequence of the present invention by recombinant methods to form a nucleic acid which is not naturally formed in nature. Such nucleic acids can encode chimeric and/or fusion proteins. Thus the heterologous nucleotide sequence can encode peptides and/or proteins which contain regulatory and/or structural properties. In another 5 such embodiment the heterologous nucleotide can encode a protein or peptide that functions as a means of detecting the protein or peptide encoded by the nucleotide sequence of the present invention after the recombinant nucleic acid is expressed. In still another such embodiment the heterologous nucleotide can function as a means of detecting a nucleotide sequence of the present invention. A heterologous nucleotide sequence can 10 comprise non-coding sequences including restriction sites, regulatory sites, promoters and the like.

The present invention also relates to cloning vectors containing genes encoding analogs and derivatives of the Stat protein, including the truncated Stat protein, of the invention, 15 that have the same or homologous functional activity as Stat protein, and homologs thereof. The production and use of derivatives and analogs related to the Stat protein are within the scope of the present invention.

Stat protein derivatives and analogs as described above can be made by altering encoding 20 nucleic acid sequences by substitutions, *e.g.* replacing a cysteine with a threonine, additions or deletions that provide for functionally equivalent molecules. Preferably, derivatives are made that have enhanced or increased functional activity relative to native Stat protein. Alternatively, such derivatives may encode soluble recombinant fragments of Stat protein such as Stat1tc having an amino acid sequence of SEQ ID NO:3.

25 Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as a *truncated Stat protein* gene may be used in the practice of the present invention. These include but are not limited to allelic genes, homologous genes from other species, which are altered by the substitution of 30 different codons that encode the same amino acid residue within the sequence, thus producing a silent change. Likewise, the truncated Stat protein derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of a truncated Stat protein including altered sequences in which functionally equivalent amino acid residues are substituted for

residues within the sequence resulting in a conservative amino acid substitution. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity, which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. Amino acids containing aromatic ring structures are phenylalanine, tryptophan, and tyrosine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Such alterations will not be expected to affect apparent molecular weight as determined by polyacrylamide gel electrophoresis, or isoelectric point.

15 Particularly preferred substitutions are:

- Lys for Arg and vice versa such that a positive charge may be maintained;
- Glu for Asp and vice versa such that a negative charge may be maintained;
- Ser for Thr such that a free -OH can be maintained; and
- Gln for Asn such that a free NH<sub>2</sub> can be maintained.

20

Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced a potential site for disulfide bridges with another Cys. A His may be introduced as a particularly "catalytic" site (i.e., His can act as an acid or base and is the most common amino acid in 25 biochemical catalysis). Pro may be introduced because of its particularly planar structure, which induces  $\beta$ -turns in the protein's structure.

The genes encoding Stat proteins, truncated Stat protein and derivatives and analogs thereof can be produced by various methods known in the art. The manipulations which 30 result in their production can occur at the gene or protein level. For example, the cloned truncated Stat protein gene sequence can be modified by any of numerous strategies known in the art (Sambrook et al., 1989, *supra*). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the production of the gene

encoding a derivative or analog of a Stat protein or a truncated Stat protein, care should be taken to ensure that the modified gene remains within the same translational reading frame as the Stat protein gene, uninterrupted by translational stop signals, in the gene region where the desired activity is encoded.

5

Additionally, the Stat or truncated Stat protein-encoding nucleic acid sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Preferably, such mutations enhance the functional activity or isolatability of the mutated truncated or native Stat protein gene product. Any technique for mutagenesis known in the art can be used, including but not limited to, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., 1978, J. Biol. Chem. 253:6551; Zoller and Smith, 1984, DNA 3:479-488; Oliphant et al., 1986, Gene 44:177; Hutchinson et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:710), use of TAB® linkers (Pharmacia), etc. PCR techniques are preferred for site directed mutagenesis (see Higuchi, 1989, "Using PCR to Engineer DNA", in *PCR Technology: Principles and Applications for DNA Amplification*, H. Erlich, ed., Stockton Press, Chapter 6, pp. 61-70).

10 The identified and isolated gene can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Examples of vectors include, but are not limited to, *E. coli*, bacteriophages such as lambda derivatives, or plasmids such as pBR322

15 derivatives or pUC plasmid derivatives, e.g., pGEX vectors, pmal-c, pFLAG, etc. The insertion into a cloning vector can, for example, be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified.

20 Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc., so that many copies of the gene sequence are generated. Preferably,

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the cloned gene is contained on a shuttle vector plasmid, which provides for expansion in a cloning cell, *e.g.*, *E. coli*, and facile purification for subsequent insertion into an appropriate expression cell line, if such is desired. For example, a shuttle vector, which is a vector that can replicate in more than one type of organism, can be prepared for 5 replication in both *E. coli* and *Saccharomyces cerevisiae* by linking sequences from an *E. coli* plasmid with sequences from the yeast 2 $\mu$  plasmid.

In an alternative method, the desired gene may be identified and isolated after insertion into a suitable cloning vector in a "shot gun" approach. Enrichment for the desired gene, 10 for example, by size fractionation, can be done before insertion into the cloning vector.

#### Expression of Stat Proteins

The nucleotide sequence coding for a Stat protein, or functional fragment, including the truncated Stat protein and the N-terminal peptide fragment of a Stat protein, derivatives or 15 analogs thereof, including a chimeric protein, thereof, can be inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. Such elements are termed herein a "promoter." Thus, the nucleic acid encoding a Stat protein of the invention or functional fragment, including the truncated Stat protein and the N-terminal 20 peptide fragment of a Stat protein, derivatives or analogs thereof, is operationally associated with a promoter in an expression vector of the invention. Both cDNA and genomic sequences can be cloned and expressed under control of such regulatory sequences. An expression vector also preferably includes a replication origin. The necessary transcriptional and translational signals can be provided on a recombinant 25 expression vector. As detailed below, all genetic manipulations described for the Stat gene in this section, may also be employed for genes encoding a functional fragment, including the truncated Stat protein and the N-terminal peptide fragment of a Stat protein, derivatives or analogs thereof, including a chimeric protein, thereof.

- 30 Potential host-vector systems include but are not limited to mammalian cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (*e.g.*, baculovirus); microorganisms such as yeast containing yeast vectors; or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the

host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.

- A recombinant Stat protein of the invention, may be expressed chromosomally, after  
5 integration of the coding sequence by recombination. In this regard, any of a number of amplification systems may be used to achieve high levels of stable gene expression (*See* Sambrook et al., 1989, *supra*).

- The cell into which the recombinant vector comprising the nucleic acid encoding Stat  
10 protein is cultured in an appropriate cell culture medium under conditions that provide for expression of Stat protein by the cell.

- Any of the methods previously described for the insertion of DNA fragments into a cloning vector may be used to construct expression vectors containing a gene consisting of  
15 appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombination (genetic recombination).

- Expression of Stat protein may be controlled by any promoter/enhancer element known in  
20 the art, but these regulatory elements must be functional in the host selected for expression. Promoters which may be used to control Stat protein gene expression include, but are not limited to, the SV40 early promoter region (Benoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes thymidine kinase  
25 promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as the  $\beta$ -lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the *tac* promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25); see also "Useful proteins from  
30 recombinant bacteria" in Scientific American, 1980, 242:74-94; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter; and the animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic

- acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver
- 10 (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58), alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Koliass et al., 1986, Cell 46:89-94), myelin basic protein gene control region which is active in oligodendrocyte
- 15 cells in the brain (Readhead et al., 1987, Cell 48:703-712), myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).
- 20 Expression vectors containing a nucleic acid encoding a Stat protein of the invention can be identified by four general approaches: (a) PCR amplification of the desired plasmid DNA or specific mRNA, (b) nucleic acid hybridization, (c) presence or absence of selection marker gene functions, and (d) expression of inserted sequences. In the first approach, the nucleic acids can be amplified by PCR to provide for detection of the
- 25 amplified product. In the second approach, the presence of a foreign gene inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to an inserted marker gene. In the third approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "selection marker" gene functions (*e.g.*,  $\beta$ -galactosidase activity,
- 30 thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. In another example, if the nucleic acid encoding Stat protein is inserted within the "selection marker" gene sequence of the vector, recombinants containing the Stat protein insert can be identified by the absence of the Stat protein gene function. In the fourth

approach, recombinant expression vectors can be identified by assaying for the activity, biochemical, or immunological characteristics of the gene product expressed by the recombinant, provided that the expressed protein assumes a functionally active conformation.

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A wide variety of host/expression vector combinations may be employed in expressing the DNA sequences of this invention. Useful expression vectors, for example, may consist of segments of chromosomal, nonchromosomal and synthetic DNA sequences. Suitable vectors include derivatives of SV40 and known bacterial plasmids, e.g., *E. coli* plasmids

- 10 col El, pCR1, pBR322, pMal-C2, pET, pGEX (Smith *et al.*, 1988, Gene 67:31-40), pMB9 and their derivatives, plasmids such as RP4; phage DNAS, e.g., the numerous derivatives of phage  $\lambda$ , e.g., NM989, and other phage DNA, e.g., M13 and filamentous single stranded phage DNA; yeast plasmids such as the  $2\mu$  plasmid or derivatives thereof; vectors useful in eukaryotic cells, such as vectors useful in insect or mammalian cells;
- 15 vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences; and the like.

- For example, in a baculovirus expression systems, both non-fusion transfer vectors, such
- 20 as but not limited to pVL941 (*Bam*H1 cloning site; Summers), pVL1393 (*Bam*H1, *Sma*I, *Xba*I, *Eco*R1, *Not*I, *Xma*III, *Bgl*II, and *Pst*I cloning site; Invitrogen), pVL1392 (*Bgl*III, *Pst*I, *Not*I, *Xma*III, *Eco*RI, *Xba*I, *Sma*I, and *Bam*H1 cloning site; Summers and Invitrogen), and pBlueBacIII (*Bam*H1, *Bgl*III, *Pst*I, *Nco*I, and *Hind*III cloning site, with blue/white recombinant screening possible; Invitrogen), and fusion transfer vectors, such
  - 25 as but not limited to pAc700 (*Bam*H1 and *Kpn*I cloning site, in which the *Bam*H1 recognition site begins with the initiation codon; Summers), pAc701 and pAc702 (same as pAc700, with different reading frames), pAc360 (*Bam*H1 cloning site 36 base pairs downstream of a polyhedron initiation codon; Invitrogen(195)), and pBlueBacHisA, B, C (three different reading frames, with *Bam*H1, *Bgl*II, *Pst*I, *Nco*I, and *Hind*III cloning site,
  - 30 an N-terminal peptide for ProBond purification, and blue/white recombinant screening of plaques; Invitrogen (220)) can be used.

Mammalian expression vectors contemplated for use in the invention include vectors with inducible promoters, such as the dihydrofolate reductase (DHFR) promoter, e.g., any

expression vector with a *DHFR* expression vector, or a *DHFR*/methotrexate co-amplification vector, such as pED (*PstI*, *SalI*, *SbaI*, *SmaI*, and *EcoRI* cloning site, with the vector expressing both the cloned gene and *DHFR*; see Kaufman, *Current Protocols in Molecular Biology*, 16.12 (1991). Alternatively, a glutamine synthetase/methionine sulfoximine co-amplification vector, such as pEE14 (*HindIII*, *XbaI*, *SmaI*, *SbaI*, *EcoRI*, and *BclI* cloning site, in which the vector expresses glutamine synthase and the cloned gene; Celltech). In another embodiment, a vector that directs episomal expression under control of Epstein Barr Virus (EBV) can be used, such as pREP4 (*BamH1*, *SfiI*, *XhoI*, *NotI*, *NheI*, *HindIII*, *NheI*, *PvuII*, and *KpnI* cloning site, constitutive RSV-LTR promoter, 5 hygromycin selectable marker; Invitrogen), pCEP4 (*BamH1*, *SfiI*, *XhoI*, *NotI*, *NheI*, *HindIII*, *NheI*, *PvuII*, and *KpnI* cloning site, constitutive hCMV immediate early gene, 10 hygromycin selectable marker; Invitrogen), pMEP4 (*KpnI*, *PvuI*, *NheI*, *HindIII*, *NotI*, *XhoI*, *SfiI*, *BamH1* cloning site, inducible methallothionein IIa gene promoter, hygromycin selectable marker; Invitrogen), pREP8 (*BamH1*, *XhoI*, *NotI*, *HindIII*, *NheI*, and *KpnI* 15 cloning site, RSV-LTR promoter, histidinol selectable marker; Invitrogen), pREP9 (*KpnI*, *NheI*, *HindIII*, *NotI*, *XhoI*, *SfiI*, and *BamHI* cloning site, RSV-LTR promoter, G418 selectable marker; Invitrogen), and pEBVHis (RSV-LTR promoter, hygromycin selectable marker, N-terminal peptide purifiable via ProBond resin and cleaved by enterokinase; Invitrogen). Selectable mammalian expression vectors for use in the invention include 20 pRc/CMV (*HindIII*, *BstXI*, *NotI*, *SbaI*, and *Apal* cloning site, G418 selection; Invitrogen), pRc/RSV (*HindIII*, *SpeI*, *BstXI*, *NotI*, *XbaI* cloning site, G418 selection; Invitrogen), and others. Vaccinia virus mammalian expression vectors (see, Kaufman, 1991, *supra*) for 25 use according to the invention include but are not limited to pSC11 (*SmaI* cloning site, TK- and β-gal selection), pMJ601 (*SalI*, *SmaI*, *AfII*, *NarI*, *BspMII*, *BamHI*, *Apal*, *NheI*, *SacII*, *KpnI*, and *HindIII* cloning site; TK- and β-gal selection), and pTKgptF1S (*EcoRI*, *PstI*, *SalI*, *AccI*, *HindII*, *SbaI*, *BamHI*, and *Hpa* cloning site, TK or XPRT selection).

Yeast expression systems can also be used according to the invention to express OB polypeptide. For example, the non-fusion pYES2 vector (*XbaI*, *SphI*, *ShoI*, *NotI*, *GstXI*, 30 *EcoRI*, *BstXI*, *BamH1*, *SacI*, *KpnI*, and *HindIII* cloning sit; Invitrogen) or the fusion pYESHisA, B, C (*XbaI*, *SphI*, *ShoI*, *NotI*, *BstXI*, *EcoRI*, *BamH1*, *SacI*, *KpnI*, and *HindIII* cloning site, N-terminal peptide purified with ProBond resin and cleaved with enterokinase; Invitrogen), to mention just two, can be employed according to the present invention.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include,

- 5 but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (*e.g.*, lambda), and plasmid and cosmid DNA vectors, to name but a few.
- 10 Vectors are introduced into the desired host cells by methods known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, lipofection (lysosome fusion), use of a gene gun, or a DNA vector transporter (see, *e.g.*, Wu et al., 1992, *J. Biol. Chem.* 267:963-967; Wu and Wu, 1988, *J. Biol. Chem.* 263:14621-14624; Hartmut et al., Canadian Patent Application 15 No. 2,012,311, filed March 15, 1990).

General Protein Purification Procedures:

Initial steps for purifying the proteins of the present invention include salting in or salting 20 out, such as in ammonium sulfate fractionations; solvent exclusion fractionations, *e.g.*, an ethanol precipitation; detergent extractions to free membrane bound proteins using such detergents as Triton X-100, Tween-20 etc.; or high salt extractions. Solubilization of proteins may also be achieved using aprotic solvents such as dimethyl sulfoxide and hexamethylphosphoramide. In addition, high speed ultracentrifugation may be used either 25 alone or in conjunction with other extraction techniques.

Generally good secondary isolation or purification steps include solid phase absorption using calcium phosphate gel or hydroxyapatite; or solid phase binding. Solid phase binding may be performed through ionic bonding, with either an anion exchanger, such as 30 diethylaminoethyl (DEAE), or diethyl [2-hydroxypropyl] aminoethyl (QAE) SEPHADEX or cellulose; or with a cation exchanger such as carboxymethyl (CM) or sulfopropyl (SP) SEPHADEX or cellulose. Alternative means of solid phase binding includes the exploitation of hydrophobic interactions *e.g.*, the using of a solid support such as phenylSEPHAROSE and a high salt buffer; affinity-binding, using, *e.g.*, placing a specific

DNA binding site of a Stat protein to an activated support; immuno-binding, using *e.g.*, an antibody to the Stat protein bound to an activated support; as well as other solid phase supports including those that contain specific dyes or lectins etc. A further solid phase support technique that is often used at the end of the purification procedure relies on size 5 exclusion, such as SEPHADEX and SEPHAROSE gels, or pressurized or centrifugal membrane techniques, using size exclusion membrane filters.

Solid phase support separations are generally performed batch-wise with low-speed centrifugations or by column chromatography. High performance liquid chromatography 10 (HPLC), including such related techniques as FPLC, is presently the most common means of performing liquid chromatography. Size exclusion techniques may also be accomplished with the aid of low speed centrifugation.

In addition size permeation techniques such as gel electrophoretic techniques may be 15 employed. These techniques are generally performed in tubes, slabs or by capillary electrophoresis.

Almost all steps involving protein purification employ a buffered solution. Unless otherwise specified, generally 25-100 mM concentrations are used. Low concentration 20 buffers generally infer 5-25 mM concentrations. High concentration buffers generally infer concentrations of the buffering agent of between 0.1-2M concentrations. Typical buffers can be purchased from most biochemical catalogues and include the classical buffers such as Tris, pyrophosphate, monophosphate and diphosphate. The Good buffers [Good, N.E., et al.,(1966) Biochemistry, 5, 467; Good, N.E. and Izawa, S., (1972) 25 Meth. Enzymol., 24, Part B, 53; and Ferguson, W.J. and Good, N. E., (1980) Anal. Biochem. 104, 300.] such as Mes, Hepes, Mops, tricine and Ches.

Materials to perform all of these techniques are available from a variety of sources such as Sigma Chemical Company in St. Louis, Missouri.

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#### Synthetic Polypeptides and Fragments Thereof

The term "polypeptide" is used in its broadest sense to refer to a compound of two or more subunit amino acids, amino acid analogs, or peptidomimetics. The subunits may be

linked by peptide bonds. In another embodiment, the subunit may be linked by other the bonds, *e.g.*, ester, ether, etc. As used herein the term "amino acid" refers to either natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics. A peptide of three or more 5 amino acids is commonly called an oligopeptide if the peptide chain is short. If the peptide chain is long, the peptide is commonly called a polypeptide or a protein.

The Stat proteins and active fragments thereof, including the truncated Stat protein of the present invention may be chemically synthesized. In addition, potential drugs that may be 10 tested in the drug screening assays of the present invention may also be chemically synthesized. Synthetic polypeptides, prepared using the well known techniques of solid phase, liquid phase, or peptide condensation techniques, or any combination thereof, can include natural and unnatural amino acids. Amino acids used for peptide synthesis may be standard Boc ( $N^{\alpha}$ -amino protected  $N^{\alpha}$ -t-butyloxycarbonyl) amino acid resin with the 15 standard deprotecting, neutralization, coupling and wash protocols of the original solid phase procedure of Merrifield (1963, J. Am. Chem. Soc. 85:2149-2154), or the base-labile  $N^{\alpha}$ -amino protected 9-fluorenylmethoxycarbonyl (Fmoc) amino acids first described by Carpino and Han (1972, J. Org. Chem. 37:3403-3409). Both Fmoc and Boc  $N^{\alpha}$ -amino 20 protected amino acids can be obtained from Fluka, Bachem, Advanced Chemtech, Sigma, Cambridge Research Biochemical, Bachem, or Peninsula Labs or other chemical companies familiar to those who practice this art. In addition, the method of the invention can be used with other  $N^{\alpha}$ -protecting groups that are familiar to those skilled in this art. Solid phase peptide synthesis may be accomplished by techniques familiar to those in the art and provided, for example, in Stewart and Young, 1984, Solid Phase Synthesis, 25 Second Edition, Pierce Chemical Co., Rockford, IL; Fields and Noble, 1990, Int. J. Pept. Protein Res. 35:161-214, or using automated synthesizers, such as sold by ABS. Thus, polypeptides of the invention may comprise D-amino acids, a combination of D- and L-amino acids, and various "designer" amino acids (*e.g.*,  $\beta$ -methyl amino acids,  $C\alpha$ -methyl amino acids, and  $N\alpha$ -methyl amino acids, etc.) to convey special properties. Synthetic 30 amino acids include ornithine for lysine, fluorophenylalanine for phenylalanine, and norleucine for leucine or isoleucine. Additionally, by assigning specific amino acids at specific coupling steps,  $\alpha$ -helices,  $\beta$  turns,  $\beta$  sheets,  $\gamma$ -turns, and cyclic peptides can be generated.

ABSTRACT INFORMATION

In a further embodiment, subunits of peptides that confer useful chemical and structural properties will be chosen. For example, peptides comprising D-amino acids will be resistant to L-amino acid-specific proteases *in vivo*. In addition, the present invention envisions preparing peptides that have more well defined structural properties, and the use 5 of peptidomimetics, and peptidomimetic bonds, such as ester bonds, to prepare peptides with novel properties. In another embodiment, a peptide may be generated that incorporates a reduced peptide bond, i.e., R<sub>1</sub>-CH<sub>2</sub>-NH-R<sub>2</sub>, where R<sub>1</sub> and R<sub>2</sub> are amino acid residues or sequences. A reduced peptide bond may be introduced as a dipeptide subunit. Such a molecule would be resistant to peptide bond hydrolysis, *e.g.*, protease 10 activity. Such peptides would provide ligands with unique function and activity, such as extended half-lives *in vivo* due to resistance to metabolic breakdown, or protease activity. Furthermore, it is well known that in certain systems constrained peptides show enhanced 15 functional activity (Hruby, 1982, Life Sciences 31:189-199; Hruby et al., 1990, Biochem J. 268:249-262); the present invention provides a method to produce a constrained peptide that incorporates random sequences at all other positions.

*Constrained and cyclic peptides.* A constrained, cyclic or rigidized peptide may be prepared synthetically, provided that in at least two positions in the sequence of the peptide an amino acid or amino acid analog is inserted that provides a chemical functional 20 group capable of crosslinking to constrain, cyclise or rigidize the peptide after treatment to form the crosslink. Cyclization will be favored when a turn-inducing amino acid is incorporated. Examples of amino acids capable of crosslinking a peptide are cysteine to form disulfides, aspartic acid to form a lactone or a lactam, and a chelator such as  $\gamma$ -carboxyl-glutamic acid (Gla) (Bachem) to chelate a transition metal and form a cross-link. Protected  $\gamma$ -carboxyl glutamic acid may be prepared by modifying the synthesis 25 described by Zee-Cheng and Olson (1980, Biophys. Biochem. Res. Commun. 94:1128-1132). A peptide in which the peptide sequence comprises at least two amino acids capable of crosslinking may be treated, *e.g.*, by oxidation of cysteine residues to form a disulfide or addition of a metal ion to form a chelate, so as to crosslink the peptide and 30 form a constrained, cyclic or rigidized peptide.

The present invention provides strategies to systematically prepare cross-links. For example, if four cysteine residues are incorporated in the peptide sequence, different protecting groups may be used (Hiskey, 1981, in *The Peptides: Analysis, Synthesis,*

Biology, Vol. 3, Gross and Meienhofer, eds., Academic Press: New York, pp. 137-167; Ponsanti et al., 1990, Tetrahedron 46:8255-8266). The first pair of cysteines may be deprotected and oxidized, then the second set may be deprotected and oxidized. In this way a defined set of disulfide cross-links may be formed. Alternatively, a pair of 5 cysteines and a pair of chelating amino acid analogs may be incorporated so that the cross-links are of a different chemical nature.

- Non-classical amino acids that induce conformational constraints.* The following non-classical amino acids may be incorporated in the peptide in order to introduce particular 10 conformational motifs: 1,2,3,4-tetrahydroisoquinoline-3-carboxylate (Kazmierski et al., 1991, J. Am. Chem. Soc. 113:2275-2283); (2S,3S)-methyl-phenylalanine, (2S,3R)-methyl-phenylalanine, (2R,3S)-methyl-phenylalanine and (2R,3R)-methyl-phenylalanine (Kazmierski and Hruby, 1991, Tetrahedron Lett.); 2-aminotetrahydronaphthalene-2-carboxylic acid (Landis, 1989, Ph.D. Thesis, University of Arizona); hydroxy-1,2,3,4-15 tetrahydroisoquinoline-3-carboxylate (Miyake et al., 1989, J. Takeda Res. Labs. 43:53-76);  $\beta$ -carboline (D and L) (Kazmierski, 1988, Ph.D. Thesis, University of Arizona); HIC (histidine isoquinoline carboxylic acid) (Zechel et al., 1991, Int. J. Pep. Protein Res. 43); and HIC (histidine cyclic urea) (Dharanipragada).
- 20 The following amino acid analogs and peptidomimetics may be incorporated into a peptide to induce or favor specific secondary structures: LL-Acp (LL-3-amino-2-propenidone-6-carboxylic acid), a  $\beta$ -turn inducing dipeptide analog (Kemp et al., 1985, J. Org. Chem. 50:5834-5838);  $\beta$ -sheet inducing analogs (Kemp et al., 1988, Tetrahedron Lett. 29:5081-5082);  $\beta$ -turn inducing analogs (Kemp et al., 1988, Tetrahedron Lett. 29:5057-5060); 25  $\alpha$ -helix inducing analogs (Kemp et al., 1988, Tetrahedron Lett. 29:4935-4938);  $\gamma$ -turn inducing analogs (Kemp et al., 1989, J. Org. Chem. 54:109:115); and analogs provided by the following references: Nagai and Sato, 1985, Tetrahedron Lett. 26:647-650; DiMaio et al., 1989, J. Chem. Soc. Perkin Trans. p. 1687; also a Gly-Ala turn analog (Kahn et al., 1989, Tetrahedron Lett. 30:2317); amide bond isostere (Jones et al., 1988, 30 Tetrahedron Lett. 29:3853-3856); tretrazol (Zabrocki et al., 1988, J. Am. Chem. Soc. 110:5875-5880); DTC (Samanen et al., 1990, Int. J. Protein Pep. Res. 35:501:509); and analogs taught in Olson et al., 1990, J. Am. Chem. Sci. 112:323-333 and Garvey et al., 1990, J. Org. Chem. 56:436. Conformationally restricted mimetics of beta turns and beta bulges, and peptides containing them, are described in U.S. Patent No. 5,440,013, issued

August 8, 1995 to Kahn.

*Derivatized and modified peptides.* The present invention further provides for modification or derivatization of a peptide of the invention. Modifications of peptides are well known to one of ordinary skill, and include phosphorylation, carboxymethylation, and acylation. Modifications may be effected by chemical or enzymatic means.

In another aspect, glycosylated or fatty acylated peptide derivatives may be prepared. Preparation of glycosylated or fatty acylated peptides is well known in the art as exemplified by the following references:

1. Garg and Jeanloz, 1985, in *Advances in Carbohydrate Chemistry and Biochemistry*, Vol. 43, Academic Press.
2. Kunz, 1987, in *Ang. Chem. Int. Ed. English* 26:294-308.
3. Horvat et al., 1988, *Int. J. Pept. Protein Res.* 31:499-507.
4. Bardaji et al., 1990, *Ang. Chem. Int. Ed. English*, 23:231.
5. Toth et al., 1990, in *Peptides: Chemistry, Structure and Biology*, Rivier and Marshal, eds., ESCOM Publ., Leiden, pp. 1078-1079.
6. Torres et al., 1989, *Experientia* 45:574-576.
7. Torres et al., 1989, *EMBO J.* 8:2925-2932.
8. Hordever and Musiol, 1990, in *Peptides: Chemistry, Structure and Biology*, *loc. cit.*, pp. 811-812.
9. Zee-Cheng and Olson, 1989, *Biochem. Biophys. Res. Commun.* 94:1128-1132.
10. Marki et al., 1977, *Helv. Chem. Acta.*, 60:807.
11. Fuji et al. 1987, *J. Chem. Soc. Chem. Commun.*, pp. 163-164.
12. Ponsati et al., 1990, *Peptides 1990*, Giralt and Andreu, eds., ESCOM Publ., pp. 238-240.
13. Fuji et al., 1987, 1988, *Peptides: Chemistry and Biology*, Marshall, ed., ESCOM Publ., Leiden, pp. 217-219.

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There are two major classes of peptide-carbohydrate linkages. First, ether bonds join the serine or threonine hydroxyl to a hydroxyl of the sugar. Second, amide bonds join glutamate or aspartate carboxyl groups to an amino group on the sugar. In particular, references 1 and 2, *supra*, teach methods of preparing peptide-carbohydrate ethers and

amides. Acetal and ketal bonds may also bind carbohydrate to peptide.

Fatty acyl peptide derivatives may also be prepared. For example, and not by way of limitation, a free amino group (N-terminal or lysyl) may be acylated, *e.g.*, myristoylated.

- 5 In another embodiment an amino acid comprising an aliphatic side chain of the structure -  $(\text{CH}_2)_n\text{CH}_3$  may be incorporated in the peptide. This and other peptide-fatty acid conjugates suitable for use in the present invention are disclosed in U.K. Patent GB-8809162.4, International Patent Application PCT/AU89/00166, and reference 5, *supra*.

10 *Phage libraries for Drug Screening.*

Phage libraries have been constructed which when infected into host *E. coli* produce random peptide sequences of approximately 10 to 15 amino acids [Parmley and Smith, Gene 73:305-318 (1988), Scott and Smith, Science 249:386-349 (1990)]. Specifically, the phage library can be mixed in low dilutions with permissive *E. coli* in low melting point

- 15 LB agar which is then poured on top of LB agar plates. After incubating the plates at 37°C for a period of time, small clear plaques in a lawn of *E. coli* will form which represents active phage growth and lysis of the *E. coli*. A representative of these phages can be absorbed to nylon filters by placing dry filters onto the agar plates. The filters can be marked for orientation, removed, and placed in washing solutions to block any  
20 remaining absorbent sites. The filters can then be placed in a solution containing, for example, a radioactive N-terminal peptide fragment of a Stat protein (*e.g.*, the fragment having the amino acid sequence of SEQ ID NO:4). After a specified incubation period, the filters can be thoroughly washed and developed for autoradiography. Plagues containing the phage that bind to the radioactive N-terminal peptide fragment of a Stat  
25 protein can then be identified. These phages can be further cloned and then retested for their ability to bind to the N-terminal peptide fragment of a Stat protein as before. Once the phages have been purified, the binding sequence contained within the phage can be determined by standard DNA sequencing techniques. Once the DNA sequence is known, synthetic peptides can be generated which represents these sequences.

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These peptides can be tested, for example, for their ability to: (1) interfere with a Stat protein binding to its DNA binding site; and (2) interfere with a truncated Stat protein binding to the DNA binding site. If the peptide interferes in the first case but does not interfere in the latter case, it may be concluded that the peptide interferes with N-terminal

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inter-protein interaction of Stat proteins.

The effective peptide(s) can be synthesized in large quantities for use in *in vivo* models and eventually in humans to prevent modulate signal transduction. It should be

- 5 emphasized that synthetic peptide production is relatively non-labor intensive, easily manufactured, quality controlled and thus, large quantities of the desired product can be produced quite cheaply. Similar combinations of mass produced synthetic peptides have recently been used with great success [Patarroyo, Vaccine 10:175-178 (1990)].

10 Antibodies to the Truncated Stat Protein

According to the present invention, the truncated Stat protein as purified from recombinant sources or produced by chemical synthesis, and derivatives or analogs thereof, including fusion proteins, may be used as an immunogen to generate antibodies that recognize the truncated Stat protein. Such antibodies include but are not limited to polyclonal, 15 monoclonal, chimeric, single chain, Fab fragments, and a Fab expression library. The anti-truncated Stat protein antibodies of the invention may be cross reactive, that is, they may recognize the truncated Stat protein derived from different natural Stat proteins such as Human Stat1 $\alpha$ , Human Stat 6 or a Drosophila Stat protein. Polyclonal antibodies have greater likelihood of cross reactivity. Alternatively, an antibody of the invention may be 20 specific for a single form of the truncated Stat, such as the Human Stat1tc having an amino acid sequence of SEQ ID NO:3.

Various procedures known in the art may be used for the production of polyclonal antibodies to the truncated Stat protein or derivative or analog thereof. For the production 25 of antibody, various host animals can be immunized by injection with the truncated Stat protein, or a derivative (*e.g.*, or fusion protein) thereof, including but not limited to rabbits, mice, rats, sheep, goats, etc. In one embodiment, the truncated Stat protein can be conjugated to an immunogenic carrier, *e.g.*, bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH). Various adjuvants may be used to increase the immunological 30 response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (*bacille Calmette-Guerin*) and *Corynebacterium parvum*.

For preparation of monoclonal antibodies directed toward the truncated Stat protein, or analog, or derivative thereof, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. These include but are not limited to the hybridoma technique originally developed by Kohler and Milstein [*Nature* 5 256:495-497 (1975)], as well as the trioma technique, the human B-cell hybridoma technique [Kozbor et al., *Immunology Today* 4:72 1983); Cote et al., *Proc. Natl. Acad. Sci. U.S.A.* 80:2026-2030 (1983)], and the EBV-hybridoma technique to produce human monoclonal antibodies [Cole et al., in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96 (1985)]. In an additional embodiment of the invention, 10 monoclonal antibodies can be produced in germ-free animals utilizing recent technology [PCT/US90/02545]. In fact, according to the invention, techniques developed for the production of "chimeric antibodies" [Morrison et al., *J. Bacteriol.* 159:870 (1984); Neuberger et al., *Nature* 312:604-608 (1984); Takeda et al., *Nature* 314:452-454 (1985)] by splicing the genes from a mouse antibody molecule specific for an truncated Stat 15 protein together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention. Such human or humanized chimeric antibodies are preferred for use in therapy of human diseases or disorders (described *infra*), since the human or humanized antibodies are much less likely than xenogenic antibodies to induce an immune response, in particular an allergic 20 response, themselves.

According to the invention, techniques described for the production of single chain antibodies [U.S. Patent Nos. 5,476,786 and 5,132,405 to Huston; U.S. Patent 4,946,778] can be adapted to produce truncated Stat protein-specific single chain antibodies. An 25 additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries [Huse et al., *Science* 246:1275-1281 (1989)] to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for a truncated Stat protein, or its derivatives, or analogs.

30 Antibody fragments which contain the idiotype of the antibody molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')<sub>2</sub> fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the F(ab')<sub>2</sub> fragment, and the Fab fragments which can be generated by treating the antibody molecule

with papain and a reducing agent.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.*, radioimmunoassay, ELISA (enzyme-linked immunosorbant assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunolectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention. For example, to select antibodies which recognize a specific epitope of the truncated Stat protein, one may assay generated hybridomas for a product which binds to the truncated Stat protein fragment containing such epitope. For selection of an antibody specific to the truncated Stat protein from a particular source, one can select on the basis of positive binding with truncated Stat protein expressed by or isolated from that specific source.

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the truncated Stat protein, *e.g.*, for Western blotting, imaging truncated Stat protein *in situ*, measuring levels thereof in appropriate physiological samples, etc. using any of the detection techniques mentioned above or known in the art.

In a specific embodiment, antibodies that agonize or antagonize the activity of truncated Stat protein can be generated. Such antibodies can be tested using the assays described *infra* for identifying ligands.

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Labels:

Suitable labels include enzymes, fluorophores (*e.g.*, fluorescein isothiocyanate (FITC), phycoerythrin (PE), Texas red (TR), rhodamine, free or chelated lanthanide series salts, especially Eu<sup>3+</sup>, to name a few fluorophores), chromophores, radioisotopes, chelating

agents, dyes, colloidal gold, latex particles, ligands (*e.g.*, biotin), and chemiluminescent agents. When a control marker is employed, the same or different labels may be used for the receptor and control marker.

- 5 In the instance where a radioactive label, such as the isotopes  $^{3}\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$ ,  $^{36}\text{Cl}$ ,  $^{51}\text{Cr}$ ,  $^{57}\text{Co}$ ,  $^{58}\text{Co}$ ,  $^{59}\text{Fe}$ ,  $^{90}\text{Y}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ , and  $^{186}\text{Re}$  are used, known currently available counting procedures may be utilized. In the instance where the label is an enzyme, detection may be accomplished by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques known in the art.

10

Direct labels are one example of labels which can be used according to the present invention. A direct label has been defined as an entity, which in its natural state, is readily visible, either to the naked eye, or with the aid of an optical filter and/or applied stimulation, e.g. U.V. light to promote fluorescence. Among examples of colored labels,

15 which can be used according to the present invention, include metallic sol particles, for example, gold sol particles such as those described by Leuvering (U.S. Patent 4,313,734); dye sole particles such as described by Gribnau et al. (U.S. Patent 4,373,932) and May et al. (WO 88/08534); dyed latex such as described by May, *supra*, Snyder (EP-A 0 280 559 and 0 281 327); or dyes encapsulated in liposomes as described by Campbell et al. (U.S.

20 Patent 4,703,017). Other direct labels include a radionucleotide, a fluorescent moiety or a  
luminescent moiety. In addition to these direct labelling devices, indirect labels  
comprising enzymes can also be used according to the present invention. Various types of  
enzyme linked immunoassays are well known in the art, for example, alkaline phosphatase  
and horseradish peroxidase, lysozyme, glucose-6-phosphate dehydrogenase, lactate  
25 dehydrogenase, urease, these and others have been discussed in detail by Eva Engvall in  
Enzyme Immunoassay ELISA and EMIT in *Methods in Enzymology*, 70, 419-439, 1980

Suitable enzymes include, but are not limited to, alkaline phosphatase and horseradish peroxidase.

Other labels for use in the invention include magnetic beads or magnetic resonance imaging labels.

In another embodiment, a phosphorylation site can be created on an antibody of the invention for labeling with  $^{32}\text{P}$ , e.g., as described in European Patent No. 0372707 (application No. 89311108.8) by Sidney Pestka, or U.S. Patent No. 5,459,240, issued October 17, 1995 to Foxwell et al.

5

- As exemplified herein, proteins, including antibodies, can be labeled by metabolic labeling. Metabolic labeling occurs during *in vitro* incubation of the cells that express the protein in the presence of culture medium supplemented with a metabolic label, such as [ $^{35}\text{S}$ ]-methionine or [ $^{32}\text{P}$ ]-orthophosphate. In addition to metabolic (or biosynthetic) 10 labeling with [ $^{35}\text{S}$ ]-methionine, the invention further contemplates labeling with [ $^{14}\text{C}$ ]-amino acids and [ $^3\text{H}$ ]-amino acids (with the tritium substituted at non-labile positions).

#### Binding Assays for Drug Screening Assays

- The drug screening assays of the present invention may use any of a number of assays for 15 measuring the stability of a protein-protein interaction, including fragments thereof, or a protein-DNA binding interaction. In one embodiment the stability of preformed DNA protein complex between a Stat protein and its corresponding DNA binding site is examined as follows: the formation of a complex between the Stat protein and a labelled oligonucleotides is allowed to occur and unlabelled oligonucleotides are added in vast 20 molar excess after the reaction reaches equilibrium. At various times after the addition of unlabelled competitor DNA, aliquots are layered on a running native polyacrylamide gel to determine free and bound oligonucleotides. In one preferred embodiment the protein is Stat1 $\alpha$ , and two different labelled DNAs are used, the natural cfos site, an example of a "weak" site, and the mutated cfos-promotor element (M67) an example of a "strong" site 25 as described below. Other examples of weak sites include those in the promoter of the MIG gene, and those in the regulatory region of the interferon- $\gamma$  gene. Other examples of strong sites include those from the promoter of the Ly6E gene or the promoter of the IRF-1 gene.
- 30 In other binding assays, an N-terminal fragment of the Stat protein is placed or coated onto a solid support. Methods for placing the N-terminal fragment on the solid support are well known in the art and include such things as linking biotin to the fragment and linking avidin to the solid support. The corresponding free N-terminal fragment is allowed to equilibrate with the bound fragments and drugs are tested to see if they disrupt

or enhance the dimer binding. Disruption leads to either a faster release of the free N-terminal fragment which may be expressed as a faster off time, and or a greater concentration of released fragment. Enhancement leads to either a slower release of the free N-terminal fragment which may be expressed as a slower off time, and or a lower

5 concentration of released fragment.

The N-terminal fragment may be labeled as described above. For example, in one embodiment radiolabeled N-terminal fragments are used to measure the effect of a drug on binding. In another embodiment the natural ultraviolet absorbance of the free N-terminal  
10 fragments is used. In yet another embodiment, a Biocore chip (Pharmacia) coated with the N-terminal fragment of a Stat protein is used and the change in surface conductivity can be measured.

Drug screening assays may also be performed in cells which are induced to contain  
15 activated STAT proteins, which are dimeric STAT proteins. Although cells that naturally encode the STAT proteins may be used, preferably a cell is used that is transfected with a plasmid encoding the STAT protein. For example transient transfections can be performed with 50% confluent U3A cells using the calcium phosphate method as instructed by the manufacturer (Stratagene). In addition the cells can also be modified to  
20 contain one or more reporter genes, a heterologous gene encoding a reporter such as luciferase, green fluorescent protein or derivative thereof, chloramphenicol acetyl transferase,  $\beta$ -galactosidase, etc. Such reporter genes can individually be operably linked to promoters comprising two weak STAT binding sites and/or a promoter comprising a strong STAT binding site. Assays for detecting the reporter gene products are readily  
25 available in the literature for example, luciferase assays can be performed according to the manufacturer's protocol (Promega), and  $\beta$ -galactosidase assays can be performed as described by Ausubel *et al.*, [in *Current Protocols in Molecular Biology*, J. Wiley & Sons, Inc. (1994)].

30 In one example, the transfection reaction can comprise the transfection of a cell with a plasmid modified to contain a STAT protein, such as a pcDNA3 plasmid (Invitrogen), a reporter plasmid that contains a first reporter gene, and a reporter plasmid that contains a second reporter gene. Although the preparation of such plasmids is now routine in the art, many appropriate plasmids are commercially available *e.g.*, a plasmid with  $\beta$ -

galactosidase is available from Stratagene.

The reporter plasmids can contain specific restriction sites in which an enhancer element having a strong STAT binding site or alternatively two tandemly arranged "weak" STAT

- 5 binding sites are inserted. In one particular embodiment, thirty-six hours after transfection of the cells with a plasmid encoding STAT-1, the cells are treated with 5 ng/ml interferon- $\gamma$  Amgen for ten hours. Protein expression and tyrosine phosphorylation (to monitor STAT activation) can be determined by *e.g.*, gel shift experiments with whole cell extracts.

10

Cells containing a STAT protein and a reporter gene that is operably linked to a promoter comprising two weak STAT binding sites can be contacted with a prospective drug in the presence of a cytokine which activates the STAT(s) of interest. The amount of reporter produced in the absence and presence of prospective drug is determined and compared.

- 15 Prospective drugs which reduce the amount of reporter produced are candidate antagonists of the N-terminal interaction, whereas prospective drugs which increase the amount of reporter produced are candidate agonists. Cells containing a reporter gene operably linked to a promoter comprising a strong STAT binding site are then contacted with these candidate drugs, in the presence of a cytokine which activates the STAT(s) of interest.

- 20 The amount (and/or activity) of reporter produced in the presence and absence of candidate drugs is determined and compared. Drugs which disrupt interactions between the N-terminal domains of the STATs will not reduce reporter activity in this second step. Similarly, candidate drugs which enhance interactions between N-terminal domains of STATs will not increase reporter activity in this second step.

25

The present invention may be better understood by reference to the following non-limiting Example, which is provided as exemplary of the invention. The following example is presented in order to more fully illustrate the preferred embodiments of the invention. It should in no way be construed, however, as limiting the broad scope of the invention.

30

#### EXAMPLE

DNA Binding of *in vitro* activated purified Stat1 $\alpha$ , Stat1 $\beta$  and truncated Stat1: Interaction

between NH<sub>2</sub> terminal domains stabilizes binding of two dimers to tandem DNA sites:

#### Introduction

To conveniently study the biochemistry of activated Stat molecules, it is necessary not  
5 only to use recombinant DNA techniques to produce large amounts of protein, but it is also necessary to phosphorylate the correct tyrosine residue and to separate the phosphorylated and nonphosphorylated proteins. The present invention teaches proteins, nucleic acids, and methods that satisfy these heretofore, unattained criteria.

10 Human Stat1 $\alpha$  and Stat1 $\beta$ , a shorter protein translated from an alternatively spliced mRNA, were produced in insect cells infected with recombinant baculovirus, thereby allowing milligram amounts of these proteins to be isolated at a time. The protease sensitivity of purified Stat1 $\alpha$  was subsequently studied. A stable truncated form of Stat1 (Stat1tc) was then characterized and produced in bacteria. Stat1 $\alpha$ , Stat1 $\beta$  and Stat1tc were  
15 quantitatively phosphorylated *in vitro* with immunoprecipitated, activated EGF-receptor kinase. The phosphoproteins were isolated in milligram quantities by a new chromatographic protocol, and the phosphorylation was shown to be on the correct tyrosine residue by mass spectroscopy of Stat1 fragments. Both the full length and the truncated phosphorylated protein dimerize and bind to DNA.

20 With the purified activated DNA binding form of Stat1 available, its DNA binding characteristics were studied. A KD of about  $1 \times 10^{-9}$  M for a variety of recognition sequences was determined. By examining the stability of labelled preformed protein/DNA complexes when challenged with unlabelled DNA, we found a very short half-life of the  
25 protein/DNA complexes. For sites that showed the maximum binding stability, we determined a half-life,  $t_{1/2}$ , of about 3 min. A more rapid exchange (half-life of <30 sec) was observed for both Stat1 $\alpha$  or Stat1tc bound to the sites that are natural "weak" binding sites in genomic DNA. Stat1 dimers (Guyer *et al.*, 1995) or dimers of Drosophila Stat protein (D-Stat) (Yan *et al.*, 1996) may interact when two nearby Stat binding sites are  
30 both occupied. The purified activated human protein behaves in a similar manner based on evidence of interaction between bound dimeric molecules in which the binding of Stat dimers to adjacent DNA binding sites was stabilized when both sites were occupied. Furthermore this proposed Stat dimer interaction is dependent on the presence of the amino terminal 131 amino acids of Stat1.

Materials and Methods

*Expression and purification of Stat1 $\alpha$  and Stat1 $\beta$ .* Nucleic acids containing sequences coding for human Stat1 $\alpha$  and Stat1 $\beta$  were amplified by PCR (primers containing respective restriction sites in addition to homologous sequence; Vent-polymerase; New England Biolabs) and the products cloned into the StuI/BglII (Stat1 $\alpha$ ) or EcoRI/KpnI (Stat1 $\beta$ ) - sites of the baculovirus transfer vector pAcSG2 (Pharmingen). Recombinant vectors were subsequently co-transfected with Baculogold baculovirus DNA (Pharmingen) into Sf9 insect cells as described (Gruenwald and Heitz, 1993). Recombinant viruses were identified by immunoblot of extracts of infected cells. For protein production Sf9 cells in suspension culture ( $0.8 \times 10^6$  cells/ml) were infected with recombinant viruses (mean of infection: 1.5) and harvested by centrifugation (1500 x g, 15 min) 50 h post infection.

The cells ( $5-8 \times 10^8$ ) were lysed in 80 ml ice cold extraction buffer [20 mM Mes, 100 mM KCl, 10 mM NaF, 10 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> pH 7.0, 10 mM NaPPi, 0.02% NaN<sub>3</sub>, 15 4 mM EDTA, 1 mM EGTA, 20 mM DTT, Complete™ protease inhibitors (Boehringer Mannheim), pH adjusted to 7.0 with 1 M Tris] with a dounce homogenizer (2 x 10 strokes). All subsequent steps were performed at 4°C unless noted otherwise. Lysates were cleared by centrifugation at 20,000 x g for 30 min. The supernatant was brought to pH 6.2 with 1 M Mes and after the addition of 0.5 vol buffer 1 (20 mM Mes, 0.02% NaN<sub>3</sub>, 20 mM DTT, pH adjusted to 6.0 with 1 M Tris) it was again centrifuged for 20 min at 25,000 x g. The resulting supernatant was loaded onto a S-Sepharose (Pharmacia) column (5 x 5.5 cm) and eluted with a linear salt gradient (50-300 mM KCl) and pH gradient (pH 6-7). Stat protein containing fractions, identified by immunoblot, were pooled, the pH adjusted to 8.0 with 1 M Tris and after the addition of 0.25 vol buffer 2 (20 mM Tris/HCl, 0.02% NaN<sub>3</sub>, 10 mM DTT, pH 8.0) loaded onto a Q-Sepharose (Pharmacia) column (2 x 9 cm). This column was developed with a linear KCl gradient from 100 mM to 300 mM KCl. Eluted Stat1 proteins were precipitated with solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to 60% saturation. The concentrated Stat proteins were dissolved in ~10 ml of buffer 3 [50 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> pH 7.2, 2 mM DTT, 1 mM EDTA, Complete™ protease inhibitors]. N-ethyl-maleimide (Sigma) was added to a final concentration of 20 mM. The alkylation reaction mixture was incubated at room temperature for 10 min and then placed on ice for another 30 min. The reaction was stopped by the addition of  $\beta$ -mercaptoethanol to 50 mM and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to 0.5 M. The reaction mixture was then loaded onto a low substituted Phenyl-Sepharose (Pharmacia) column (2 x 15 cm)

equilibrated in buffer 4 (20 mM Tris/HCl, 2 mM DTT, pH 7.4) + 0.5 M ammonium sulfate and the Stat proteins were eluted with decreasing  $(\text{NH}_4)_2\text{SO}_4$  in buffer 4. (The Stat proteins eluted at about 300 mM salt). Fractions of interest were pooled, concentrated by centriprep 50 (Amicon) to about 10 mg/ml and applied to a SUPERDEX 200 column (XK 5 16, Pharmacia) equilibrated in buffer 5 (20 mM Hepes/HCl, 0.02%  $\text{NaN}_3$ , 2 mM DTT, 0.3 M KCl, pH 7.2). Fractions containing  $\text{Stat}1\alpha$  or  $\text{Stat}1\beta$  were pooled. Both  $\text{Stat}1\alpha$  and  $\text{Stat}1\beta$  eluted very early, e.g. with a volume typical for globular proteins of  $M_r$  350 kD. The pooled fractions were then concentrated by ultrafiltration to approximately 20 mg/ml and quick frozen on dry ice. The purified proteins were stored at -70°C. All buffers used 10 during protein purification were chilled, thoroughly degassed and flushed with  $\text{N}_2$  before use.

*Expression and purification of  $\text{Stat}1\text{tc}$ .* The portion of the human  $\text{Stat}1$  gene encoding residues 132-713 was amplified by PCR (Vent-Polymerase). The following primers were 15 used: 5'-dGGGAATTCCATATGAGCACAGTGATG-TTAGACAAAC and 5'-dCGGATCCATTAGTGAACTTCCAGACACAGAAATC (restriction sites underlined). The product was cloned into the NdeI/BamHI sites of the pET20b expression vector (Novagen). N-terminal sequencing revealed the absence of the methionine residue introduced with the NdeI restriction site. Growth and induction of transformed *E. coli* 20 [BL21DE3 (pLysS)] was as described (Studier and Moffatt, 1986). About 50% of the induced protein remained soluble and was subsequently isolated. Cells were collected by centrifugation (20 min; 4°C; 20,000g) and resuspended in ice cold extraction buffer (100 ml/30 g cells; 20 mM Hepes/HCl, 0.1 M KCl, 10% Glycerol, 1mM EDTA, 10 mM MnCl<sub>2</sub>, 20 mM DTT, 100 U/ml DNase I (Boehringer Mannheim), Complete™ protease 25 inhibitor, pH 7.6). Cells were lysed by three cycles of freeze/thawing. Lysis was continued at 4°C while stirring slowly for 1 h. The lysate was centrifuged for 20 min at 22,000 x g at 4°C. Polyethylenimine (0.1% final; Sigma) was added to the supernatant, the solution gently mixed and centrifuged for 15 min at 15,000 x g. All subsequent steps 30 were performed in the cold (4°C) unless stated otherwise. The supernatant containing soluble  $\text{Stat}1\text{tc}$  was precipitated with saturated ammonium sulfate solution (ultrapure; Gibco) in two steps (0-35%; 35-55% saturation final). The 35-55% pellet was redissolved in 20 ml of buffer 3 (see above) and alkylated as described above. The reaction was stopped by the addition of  $\beta$ -mercaptoethanol to 50 mM and solid ammonium sulfate to 0.9 M. The mixture was loaded onto a Fast Flow Phenyl-SEPHAROSE column (low

substituted, 2 x 15 cm) that had been equilibrated in buffer A (50 mM Tris/HCl, 1 mM EDTA, 0.02% NaN<sub>3</sub>, 2 mM DTT, pH 7.4) + 0.9 M ammonium sulfate. After washing the column a linear gradient from 0.9 M to 0.05 M ammonium sulfate in buffer A was applied. Stat1tc eluted at about 0.5 M salt and the Stat1tc containing fractions were pooled

5 and dialysed overnight against 2 x 4 liters of buffer B (40 mM Mes/NaOH, 10% Glycerol, 0.5 mM EDTA, 0.02% NaN<sub>3</sub>, pH 6.5) + 140 mM KCl. This material was loaded onto a S-Sepharose column (5 x 5.5 cm) and a linear 500 ml gradient of buffer B containing 140 mM to 300 mM KCl was applied. The protein eluted in at approximately 220 mM KCL. Fractions of interest were collected and dialysed against 3 liters of buffer

10 C (50 mM Tris/HCl, 10% Glycerol, 2mM DTT, pH 8) + 50 mM KCl with one change of buffer. The protein solution (in buffer C + 50 mM KCl) was then applied to a Q-Sepharose column (2 x 9 cm) and bound proteins were eluted with a linear gradient from 50 to 300 mM KCl in buffer C. Fractions with Stat1tc were combined and precipitated with solid ammonium sulfate to 55% saturation. At this stage the 95% pure preparation

15 could be stored at -20°C until subjected to *in vitro* phosphorylation (see below) or was directly loaded onto a SUPERDEX 200 gel filtration column (XK 16; Pharmacia). In this case the precipitated protein was dissolved in about 2 ml of 10 mM Hepes/HCl, 100 mM KCl, 2 mM DTT, 0.5 mM EDTA, pH 7.4 and gel filtrated in this buffer. Stat1tc eluted in a symmetrical peak and was concentrated to about 20 mg/ml (Centriprep 50), quick frozen

20 on dry ice and stored at -70°C. Typically yields of 40-50 mg (greater than 98% pure as judged by Coomassie stain and mass spectroscopy) Stat1tc from 6 liters of starting culture could be obtained.

*Determination of protein concentrations.* Purified proteins were quantitated by UV

25 spectroscopy. The extinction coefficient  $\epsilon$  in a 1 cm path length for a 1 mg/ml solution of protein can be estimated by the formula  $[(5700 \times W + 1300 \times Y)/M_r]$  with W = number of tryptophans; Y = number of tyrosines and M<sub>r</sub> = molecular weight (Cantor and Schimmel, 1980). The following extinction values (mM<sup>-1</sup>cm<sup>-1</sup>) were used: Stat1 $\alpha$ :  $\epsilon$  = 1.25; Stat1 $\beta$ :  $\epsilon$  = 1.31; Stat1tc:  $\epsilon$  = 1.27.

30

*Proteolytic digestion of Stat1 $\alpha$  and amino-terminal sequencing of fragments.* Proteinase K and subtilisin (Sigma) digests of purified Stat1 $\alpha$  were carried out for 30 minutes on ice. The protein was digested at the concentration of 4.5  $\mu$ M in 50  $\mu$ l of cleavage buffer which contained 20 mM Hepes/HCl, 50 mM ammonium sulfate, and 10 mM MgCl<sub>2</sub>, pH 7.4.

Reactions were stopped by the addition of PMSF (2 mM final) and SDS-sample buffer. The proteolysis was resolved on a 10% or 16.5% SDS PAGE gel, which was either stained with Coomassie blue or electro-transferred onto a PVDF membrane(Immobilon P<sup>SQ</sup>; Millipore). Sequencing of the amino terminus of the 65 kDa protease resistant Stat1 $\alpha$  fragment was performed as described by LeGendre and Matsudaira, (1988). Amino terminal sequence analysis was performed by the Protein /DNA facilities at The Rockefeller University.

*Cyanogen bromide and Endoproteinase AspN digests with mass spectrometric peptide analysis.* Cyanogen bromide (Sigma) digests were performed on 90 pmol of recombinant protein in 50% formic acid at 25°C in the dark. Endoproteinase AspN (sequencing grade; Boehringer Mannheim) digests were carried out on 100-150 pmol of protein in either 25 mM Tris/HCl (pH 7.5) or 10 mM ammonium phosphate buffer (pH 8) with 150 mM KCl at 25°C. The protease:protein ratio was 1:50 by weight, e.g., 0.2  $\mu$ g: 10  $\mu$ g. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) was used to evaluate the peptide fragments. Aliquots (0.5  $\mu$ l) of the digest were taken at various intervals (1 min to 7 hours), directly mixed into the MALDI-MS matrix solution (Cohen and Chait, 1996), and subject to MALDI-MS analysis in a procedure reported earlier (Cohen *et al.*, 1995).

*Preparation of EGF-receptor kinase and in vitro phosphorylation of Stat proteins.* Human carcinoma A431 cells were grown to 90% confluence in 150 mm diameter plates in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum (Hyclone). Cells were washed once with chilled PBS and lysates were prepared in 1 ml ice cold lysis buffer (10mM Hepes/HCl, 150 mM NaCl, 0.5% Triton X-100, 10% Glycerol, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM EDTA, Complete<sup>TM</sup> protease inhibitors, pH 7.5). After 10 min on ice, the cells were scraped, vortexed and dounce homogenized (5 strokes). The lysates were cleared by centrifugation at 4°C for 20 min at top speed in an Eppendorf microfuge and stored at -70°C until needed. Immediately before use 1 volume of the lysate was diluted with 4 volumes of the lysis buffer ("diluted lysate").

EGF-receptor precipitates were obtained by incubating 5 ml of diluted lysate with 50  $\mu$ g of an anti-EGF-receptor monoclonal antibody directed against the extracellular domain. After 2 hours of rotating the sample at 4°C, 750  $\mu$ l of Protein-A-agarose (50% slurry;

Oncogene Science) was added, and the incubation was allowed to proceed, while rotating, for another 1 hour. Agarose beads containing the EGF-receptor immunoprecipitates were then washed 5 times with lysis buffer and finally twice with storage buffer (20% Glycerol, 20 mM Hepes/HCl, 100 mM NaCl, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>). Precipitates from 5 ml diluted lysate were dissolved in 0.5 ml storage buffer, flash frozen on dry ice and stored at -70°C.

5 Immediately before an *in vitro* kinase reaction the Protein-A-agarose bound EGF-receptor from 5 ml dilute lysate was washed once with 1x kinase buffer (20 mM Tris/HCl, 50 mM KCl, 0.3 mM Na<sub>3</sub>VO<sub>4</sub>, 2 mM DTT, pH 8.0) *plus* 50 mM KCL and then dissolved in 0.4 ml (total volume) of this buffer. Afterwards the washed EGF-receptor precipitate was

10 incubated on ice for 10 min in the presence of a final concentration of mouse EGF of 0.15 ng/μl. Phosphorylation reactions were carried out in Eppendorf tubes in a final volume of 1 ml. To the pre-incubated kinase preparation the following was added: 60 μl 10x kinase buffer, 20 μl 0.1 M DTT, 50 μl 0.1 M ATP, 4 mg Stat protein (SUPERDEX 200 eluate for Stat1α and Stat1 β; ammonium sulfate pellets dissolved in [20 mM Tris /HCl, pH 8.0]

15 for Stat1tc), 10 μl 1M MnCl<sub>2</sub> and dH<sub>2</sub>O to 1 ml. The reaction was allowed to proceed for 15 hours at 4°C. After 3 hours an additional 15 μl of 0.1 M ATP was added.

*Separation of phosphorylated from unphosphorylated Stat proteins.* The *in vitro* kinase reaction mixture (see above) was freed from EGF-receptor bound to agarose beads by spinning through a plug of siliconized glass wool at the bottom of a pierced Eppendorf tube. The glass wool was washed with 0.5 ml HA-buffer (20 mM Tris/HCl, 1mM EDTA, 2 mM DTT, pH 8.0) and the pooled volumes loaded onto a heparin agarose (Biorad) column (1.5 x 7 cm). The column was washed with 50 ml HA-buffer, and then the bound Stat proteins were eluted with two consecutive 50 ml volumes of HA-buffer + 150 mM KCl and then HA-buffer + 400 mM KCl. Unphosphorylated proteins (eluted with 150 mM KCl) were concentrated by ultrafiltration to about 10 mg/ml, flash frozen on dry ice and stored at -70°C. Phosphorylated Stat1α and Stat1β was concentrated to 1mg/ml. Glycerol was added to 50% (vol/vol) and the material was stored at -20°C. Phosphorylated Stat1tc was brought to a concentration of about 15 mg/ml and run on a SUPERDEX 200 columns

20 under the conditions described above for the native protein. The gel filtered phosphorylated Stat1tc was pooled, concentrated to approximately 20 mg/ml, flash frozen on dry ice and stored at -70°C.

*Electrophoretic mobility shift assays (EMSA).* A 12.5 μl reaction volume contained DNA

binding buffer (20 mM Hepes/HCl, 4% Ficoll, 40 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM CaCl<sub>2</sub>, 1 mM DTT) radiolabelled DNA (see below) at a final concentration of 1 x 10<sup>-10</sup> M unless stated otherwise, 50 ng dIdC, 0.2 mg/ml BSA (Boehringer Mannheim), and the indicated amount of purified phosphorylated Stat1. The reaction volume was mixed and  
 5 then incubated at room temperature. The time necessary to reach equilibrium was assessed by EMSA [(Stone *et al.*, 1991)]. For all DNA fragments tested, equilibrium turned out to be fully established at the earliest timepoint that can be determined by this technique (30 sec). Therefore incubation periods of 5-15 minutes were chosen. Reaction products were loaded onto a 4% polyacrylamide gel (1.5 mm thick) containing 0.25 x Tris-borate-EDTA  
 10 which had been pre-run at 20V/cm for 2 hours at 4°C. Electrophoresis was continued for 60 minutes at 4°C. Gels were dried and exposed to X-ray film and quantitated by a Molecular Dynamics PhosphoImager.

*Binding site oligonucleotides.* Single-stranded oligonucleotides that were purified on the  
 15 basis of trityl affinity were obtained from The Great American Gene Company (Ransom Hill). Oligonucleotides longer than 30 nucleotides were further purified on 6% sequencing gels and DNA recovered by soak elution and ethanol precipitation. Nucleic acid concentrations were determined by absorbance at 260 nm using the calculated molar extinction coefficient for each oligonucleotide (corrected for the hyperchromic effect).  
 20 Complementary oligonucleotides at a concentration of 1 pmol/μl were hybridized for 3 hours after thermal denaturation in 5 mM Tris/HCl, 50 mM KCl, 10 mM MgCl<sub>2</sub>, pH 8.0. One pmol of synthetic duplex molecule was labelled to high specific activity by the Klenow fill-in reaction (0.5 mM dATP (and 0.5 mM dCTP for S1), 100 μCi [ $\alpha^{32}\text{P}$ ] dGTP (3000 Ci/mmol; 10mCi/ml; and [ $\alpha^{32}\text{P}$ ] dTTP for S1; Du Pont), 5 Units of Exo Klenow  
 25 enzyme (New England Biolabs)) and rendered completely double-stranded with a 0.5 mM dGTP (and 0.5 mM dTTP for S1) cold chase. Unincorporated nucleotides were removed by gel filtration (spin quant columns; Pharmacia) in 10 mM Tris/HCl, 100 mM NaCl, 1 mM EDTA, pH 8.0. Labelled oligonucleotides were stored at 4°C.  
 30 The following duplex DNA fragments with protruding 5'-TCC (except for S1 which has 5'-GATC) were used (the core recognition sequence is underlined): cfosWT 5'-dGTATTCCCCGTCAATGCA-3'; Ly6 E 5'-dGTATTCCCTGTAAGATCT-3'; cfosM67 5'-dGATTCCCCGTAAATCAT-3'; S1 5'-dGTTGTCCGGGAAAAGG-3'; 2x cfosWT (10 bp spacing) 5'-dAGTCAGTTCCCGTCAATGCATCAGGTTCCCGTCAATGCAT-3';

- 2x cfosWT (5 bp spacing) 5'-dAGTCAGTTCCCGTCAATGAGTTCCCGTCAATGCA-3';  
 2x cfosWT (15 bp spacing)  
 5'-dAGTCAGTTCCCGTCAATGATCGCTACAGAGTTCCCGTCAAGCA-3'; 2x  
cfosWT (inverted repeat)  
 5 5'-dAGTCATTCCCGTCAATGCATCAGTTGACGGGAAAGTAGT-3'.

*Dissociation rate determination.* Under the reaction conditions described above, each oligonucleotide (at  $2 \times 10^{-9}$  M or otherwise stated) was mixed with  $0.55 \times 10^{-9}$  M dimer of purified phosphorylated Stat1 protein. The reaction volume was scaled up to 100  $\mu$ l. The 10 reaction was incubated for 5-15 min at room temperature and for time zero, an aliquot (10  $\mu$ l) was removed and loaded directly onto a pre-run polyacrylamide gel (see above). Afterwards, a 100 x molar excess of homologous unlabelled DNA (in less than 1% of the reaction volume) was added. At subsequent time points (indicated in Figures 5B, 6 and 7) 10  $\mu$ l aliquots were withdrawn and also loaded onto the running gel (at 10 V/cm). After 15 entering the final time point (after 30-45 min), electrophoresis was continued at 20 V/cm until the unbound labelled DNA-fraction reached the bottom of the gel. Gels were dried, exposed to X-ray film and labelled protein/DNA complexes and unbound labelled DNA were quantitated as described above. The half life was determined from a semi-log plot of the numerical data (shifted radioactivity over shifted radioactivity at time zero versus time). For many sequences studied, the half life was too short (>30 sec) to be 20 determined by EMSA. All experiments were performed at least twice with the different oligonucleotides.

*Determination of apparent equilibrium constants for protein: DNA interactions.* A fixed 25 quantity of  $^{32}$ P labelled oligonucleotide varied between  $1 \times 10^{-10}$  M and  $5.6 \times 10^{-10}$  M in three separate experiments, was titrated against a standard protein dilution series (common to all oligonucleotides tested) in a volume of 12.5  $\mu$ l under the reaction conditions described above. Numerical data were used to construct a standard binding curve from which the free dimer concentration, when 50% of the probe is shifted, could be 30 determined.

#### Results:

*Production by recombinant techniques and purification of Stat1:* cDNA encoding Stat1 $\alpha$  or Stat1 $\beta$  was inserted in baculovirus transfer vector (pAcSG2) and co-transfected with

modified linearized AcPNV baculovirus DNA to produce virus particles. Insect cells (Sf9 cells) infected with the respective recombinant baculovirus produced a 91 kDa protein and a 84 kDa protein that could be identified with an antibody raised against Stat1 by Western blot analysis. These proteins were purified (Fig. 1A) through the steps indicated in Table 5 1.

**Table I**Purification of Stat 1 $\alpha/\beta$ 

	STEP	VOLUME (ml)	PROTEIN (mg)
I	Crude Extract <sup>a</sup>	80	550 <sup>b</sup>
II	S-SEPHAROSE	120	30 <sup>b</sup>
III	Q-SEPHAROSE	30	12 <sup>b</sup>
IV	Ammonium Sulfate	1	8 <sup>b</sup>
V	Alkylation	10	8 <sup>b</sup>
VI	Phenyl-SEPHAROSE	25	6 <sup>c</sup>
VII	SUPERDEX	3	5 <sup>c</sup>

<sup>a</sup> Following precipitation at pH 6.2 from  $5 \times 10^8$  cells.

<sup>b</sup> Protein concentrations were determined by the method of a dye-binding assay (Bradford, 1976) using bovine serum albumin as the protein standard.

<sup>c</sup> Protein determined by ultraviolet light absorbance as described in METHODS.

Stat1 $\alpha$  is 750 amino acids long. Stat1 $\beta$  is a product of a differentially spliced mRNA which encodes a protein 712 amino acids long (Schindler *et al.*, 1992; Yan *et al.*, 1995). It is known that both Stat1 $\alpha$  and 1 $\beta$  can be phosphorylated on a single tyrosine, residue 701. *In vivo*, both forms of the protein dimerize upon phosphorylation, and then 5 translocate to the nucleus to bind specific DNA sites (Shuai *et al.*, 1992; Shuai *et al.*, 1993a).

- The purified Stat1 $\alpha$  was digested with several proteolytic enzymes to determine whether the protein could be divided into functional domains. Both subtilisin and proteinase K 10 produced two major digestion products (Fig. 1B), the largest of which migrated on SDS polyacrylamide gel electrophoresis with an estimated size of 65 kDa, as compared with the full length protein of 91 kDa. (Cleavage products of approximately 40 and 30 kDa were also seen). The 65 kDa product had an N-terminal sequence of XTVMLDKQEKE indicating that it resulted from cleavage between residues 131 and 132 of the full length 15 protein. A single prominent smaller fragment of about 16 kDa was also observed. This fragment was the only one generated that retained reactivity with an antibody raised against the amino terminus of Stat1. The shorter 16 kDa fragment was therefore identified as an N-terminal fragment of the molecule.
- 20 The major proteolytic cleavage fragment, which was also the longest, began at residue 132. This fragment was poorly recognized by an antibody to the carboxyl terminal 38 amino acids of Stat1 $\alpha$  which indicated an additional cleavage near the carboxyl terminus. A bacterial expression clone encoding residues 132-713 was prepared since this fragment was shown to be resistant to further proteolysis (above), and Stat1 $\beta$ , which terminates at 25 residue 712, is known to be active form of the protein *in vivo*. The product, Stat1 (132-713) or Stat1tc, was expressed in relatively large quantities in *E. coli* and a major fraction of the protein proved to be soluble. Stat1tc was purified to homogeneity (Fig. 1A and as in the Materials and Methods, above). The recombinant truncated Stat protein of the present invention appears to be a unique form of Stat protein, since the Stat fragments 30 listed in Table II were found to essentially accumulate entirely in inclusion bodies.

9620TTTQD90E9160

**Table II**Solubility of Recombinant Stat 1 Fragments

AMINO TERMINUS	CARBOXYL TERMINUS	SOLUBLE
132	713	YES
200	713	NO
250	713	NO
300	713	NO
370	713	NO
420	713	NO

The expression vectors for the nucleic acids coding the amino acid sequences for the protein fragments of Stat1, listed above, were constructed and expressed as described in the METHODS for the truncated protein Stat1, Stat1tc. The sequences are based on the Stat1 $\alpha$  as described above. The positive (YES) denotation for being soluble, is indicative of significant quantities of the corresponding protein fragment being free of the inclusion bodies. As can be seen from the table, only the truncated Stat protein of the present invention (132-713) was found to occur free of inclusion bodies in significant quantities.

*Aggregation of native proteins:* It appeared possible that aggregation of the protein occurred since purified Stat1 $\alpha$ , Stat1 $\beta$  and Stat1tc eluted in peaks with broad leading shoulders, during gel filtration. Thiol crosslinking was suspected as the cause, since the preparation had aggregates that migrated with an apparent molecular mass corresponding

- 5 to dimers and higher order oligomers when run under non-reducing conditions on a denaturing polyacrylamide gel (not shown). Accordingly, to block the reactive thiols, the cell extracts (from baculovirus infected Sf9 cells for Stat1 $\alpha$  and transformed *E. coli* for Stat1tc) were incubated with N-ethyl maleimide (NEM) to test if the modification of the cysteine residues: (1) could prevent the aggregation, and (2) whether such modification  
10 would lead to a non-aggregated protein preparation that retained its functional properties. The procedure worked unexpectedly well and this alkylation step became part of the purification procedure (Table 1).

The purified protein was cleaved with cyanogen bromide and Endoprotease Asp-N. Mass spectrometric analysis of the resulting peptides showed that cysteines 155, 440, and 492 were alkylated by the NEM treatment, whereas two other cysteines were not (Cys 552 and Cys 577). The NEM treatment did not affect any of the subsequent experiments (e.g., DNA binding, see Fig. 3B) and was adopted as the standard preparation of a homogeneous protein.

- 20
- In vitro phosphorylation of Stat1 $\alpha$ , Stat1 $\beta$  and truncated Stat1 by the EGF-receptor.* The *in vivo* activated DNA binding form of Stat1 is phosphorylated on tyrosine 701 when isolated from mammalian cells treated with ligands that activate either JAK kinases or transmembrane receptor kinases (Shuai *et al.*, 1992; Shuai *et al.*, 1993b). EGF-receptor kinase activity was achieved with immunoprecipitates of membrane preparations from cultured human A431 cells that express  $5 \times 10^6$  EGF-receptors per cell (Yarden *et al.*, 1985; Quelle *et al.*, 1995). These membrane preparations were used as the source of enzyme to catalyze the tyrosine phosphorylation of Stat1 and the truncated Stat1.
- 30 As detailed above, *in vivo*, Stat1 $\alpha$  is phosphorylated on a specific tyrosine residue (Tyr701). The resulting phosphorylated form of the protein runs at a slightly slower rate during polyacrylamide gel electrophoresis, in comparison to the nonphosphorylated form (Shuai *et al.*, 1992). This same change in mobility was observed after purified Stat1 $\alpha$  was treated *in vitro* with EGF-receptor kinase preparations. In addition, when the enzymatic

reaction was carried out in the presence of  $^{32}\text{P}\gamma\text{ATP}$ , the slower running protein was found to contain  $^{32}\text{P}$  (Figure 2A). Similar results were obtained for the *in vitro* phosphorylation of Stat1 $\alpha$ . However, it was clear that not all of the Stat1 protein was phosphorylated (Figure 2A). Although subsequent experiments yielded somewhat higher amounts of phosphorylation, the percentage of Stat protein that was phosphorylated never exceeded 75%.

Therefore a method of separating phosphorylated from nonphosphorylated Stat protein was required. Although the phosphorylated protein forms a dimer, this dimer elutes in a peak strongly overlapping the elution peak of the corresponding nonphosphorylated monomer. Therefore, alternative means was required. After many unsuccessful attempts using various chromatography procedures, step-wise elution of the protein mixture bound to heparin agarose proved surprisingly successful (Figure 2B). This novel procedure resulted in a separation of two peaks containing Stat proteins (eluted in steps of 150 mM and 400 mM KCl). The tyrosine phosphorylated protein (Figure 2B) which, in addition, had DNA binding activity, was present in the second of these two chromatographic peaks.

To determine the purity of the isolated material and to analyze whether the correct tyrosine residue was phosphorylated, both purified, unphosphorylated (*i.e.*, protein not reacted with EGF-receptor) and phosphorylated protein (*i.e.*, protein obtained from the chromatographic peak containing phosphotyrosine from the heparin agarose column described above) were subjected to Endoprotease Asp-N digestion and the resulting peptide fragments analyzed by mass spectrometry (Figure 2C). Phosphorylation increases the molecular mass of an unphosphorylated fragment by 80 daltons, that is, comparison of the Asp-N fragments of phosphorylated versus unphosphorylated Stat's showed an 80 dalton shift of the fragment 694 - 720 (Figure 2C), demonstrating that *in vitro* phosphorylation by EGF-receptor kinase occurred exclusively on the single tyrosine residue that is phosphorylated in the cell. In addition, the bottom panel of Figure 2C demonstrates the absence of unphosphorylated Tyr 701 in the purified EGF-receptor kinase-treated protein.

*Both in vitro phosphorylated Stat1 $\alpha$  and Stat1 $\alpha$  bind specific DNA fragments:*

Electrophoretic mobility shift assays (EMSA) (Fried and Crothers, 1981; Garner and Revzin, 1981) were used to test DNA binding of tyrosine phosphorylated Stat1 $\alpha$  and

Stat1tc. Both proteins were found to bind to all tested labelled deoxyoligonucleotides known from earlier studies to bind Stat1 (the oligo cfosWT is illustrated in Figure 3A). The bound complexes were not affected by N-ethyl maleimide indicating that alkylation of cysteine does not affect DNA binding (Fig. 3B). This result is consistent with earlier experiments showing that ISGF3 $\alpha$ , now known to be a Stat1:2 heterodimer, is not affected by NEM treatment (Levy *et al.*, 1989). In addition, the DNA binding ability of homodimeric phosphorylated Stat1 $\alpha$  or its truncated form was highly resistant to up to 2 Molar monovalent salt concentrations.

- 10 *Strength of Stat1 binding to DNA and estimation of dissociation rates.* We next used the EMSA assay to obtain an estimate of the binding affinity of Stat1 $\alpha$  and Stat1tc to DNA. Both forms of the protein behaved identically when using a fixed amount of deoxyoligonucleotide and increasing protein concentrations (Figure 4). A  $K_{eq}$  of approximately  $1 \times 10^{-9}$  M was estimated from this data for both proteins when the bound 15 and unbound fraction of DNA was compared as a function of protein concentration. This is in the affinity range for transcription factors in general which have been reported to have a  $K_{eq}$  between  $10^{-9}$  and  $10^{-12}$  M for proteins with the highest affinity for their cognate DNA sites (Riggs *et al.*, 1970; Affolter *et al.*, 1990). The same results were obtained with several different oligonucleotides, the Ly6E and cfosWT Stat binding sites, which are 20 "weak" binding sites, and "strong" sites, such as the selected optimum site, S1 (Horvath *et al.*, 1995) and a mutated cfos sequence (M67 site; Wagner *et al.*, 1990). ["Strong" and "weak" in this context refer to experiments with cell extracts containing activated Stat1 which binds more of some oligonucleotides (strong) than others (weak).]
- 25 The stability of preformed DNA protein complexes were examined by the following method: the formation of a complex between protein and labelled oligonucleotides is allowed to occur and unlabelled oligonucleotides are added in vast molar excess after the reaction reaches equilibrium. At various times after the addition of unlabelled competitor DNA, aliquots are layered on a running native polyacrylamide gel to determine free and 30 bound oligonucleotides. This type of experiment was carried out with both Stat1 $\alpha$ , and Stat1tc, and with two different labelled DNAs, the natural cfos site, an example of a "weak" site, and the mutated cfos-promotor element (M67) an example of a "strong" site.

With the "weak" site, the "off" time was so short that the addition of unlabelled

nucleotides for as little as 30 seconds removed all preformed protein DNA complexes (Figure 5B, Stat1 $\alpha$  shown in the left panel). With the "strong" site, the preformed labelled complexes were displaced more slowly, the t<sub>1/2</sub> is estimated to be 3 minutes (Figure 5B, right panel employed Stat1tc). In these experiments there was no difference  
5 between Stat1 $\alpha$  and Stat1tc.

*Stat binding to tandem DNA sites: Evidence for stabilized promotor occupancy through protein:protein interactions of Stat1 $\alpha$  and Stat1 $\beta$  versus Stat1tc.*

Two recent reports on promoters of genes dependent for transcription on Stat proteins  
10 have indicated that two neighboring Stat binding sites are required for maximal transcriptional stimulation. In one of these reports the human mig gene promoter was found to have two weak Stat1 binding sites within 25 bp, neither of which alone conferred IFN- $\gamma$  transcriptional activation while both sites together did so. Moreover the active element formed complexes with Stat1 protein that migrated more slowly than Stat1 dimers  
15 bound to DNA. The authors suggested that interaction between Stat homodimers might occur in the complexes (Guyer *et al.*, 1995). In addition, we recently reported two D-Stat binding sites were found in the segment of the even-skipped promoter that directs stripe 3 formation in Drosophila embryos; both sites were required for maximum stripe 3 expression (Yan *et al.*, 1996).

20 With the present demonstration that Stat1 $\alpha$  protein indeed does have such a rapid off-time, especially on natural "weak" binding sites, the binding of activated protein to oligonucleotides containing two weak DNA binding sites was investigated. The experiments were carried out with both Stat1 $\alpha$  and Stat1tc and a labelled oligonucleotide  
25 containing a variety of arrangements of two "weak" binding sites. With two binding sites present in tandem on the same DNA fragment and at a moderately high concentration of protein ( $0.55 \times 10^{-9}$  M), Stat1 $\alpha$  and Stat1tc each formed both a homodimer complex and an additional complex that migrated more slowly [2x (dimeric)]. The mobility of this slower moving complex suggested occupation of both DNA binding sites, indicating one  
30 DNA molecule with two Stat dimers bound to it (Figure 6A, time zero). When such complexes were challenged for various times with an excess of unlabelled oligonucleotide, both the dimeric and [2x (dimeric)] complexes were dispelled but with different kinetics for Stat1 $\alpha$  and Stat1tc. The Stat1tc showed almost immediate displacement (less than one minute) of both dimeric and [2x (dimeric)] complexes (Figure 6A, left). In contrast,

whereas as anticipated, the Stat1 $\alpha$  homodimer also disappeared quickly, the [2x (dimeric)] complex required more than 30 min for partial displacement, indicating a significant increase in stability of this larger complex with the full length proteins.

- 5 These results suggested that when Stat1 $\alpha$  is bound at tandem binding sites, protein:protein interactions occur that require the presence of the amino and/or carboxyl terminal domain of Stat1 $\alpha$  to form the more stable DNA:protein complexes. To examine this question we compared Stat1tc in the chase assay with the Stat1 $\beta$  protein, which only lacks the C-terminal domain. As shown in Figure 6B, Stat1 $\beta$  exhibits the same behavior as the full  
10 length protein, indicating involvement solely of the amino terminal region (between amino acids 1 and 131) in stabilizing the [2x (dimeric)] complexes.

We then tested the importance of the orientation and the spacing of the two Stat binding sites within the synthetic oligonucleotides. First the DNA sites that exhibited stabilization  
15 in [2x (dimeric)] binding were changed from tandem ( $\rightarrow \rightarrow$ ) to inverted ( $\rightarrow \leftarrow$ ), keeping the spacing at 10 basepairs (bp) between the two binding sites. While both oligonucleotides were capable of binding two dimers (with the tandem binding sites in inverted orientation showing much less of the [2x (dimeric)] complex even at relatively high protein to DNA ratio), the inverted sites showed no increased stability when  
20 challenged with unlabelled oligonucleotide (Fig. 7A).

Oligonucleotides with tandem binding sites spaced by 5 or 15 bp were prepared to compare with the original oligonucleotide with 10 bp spacing. The oligonucleotide with a 15 bp spacing behaved indistinguishably from the one with 10 bp spacing, while the  
25 oligonucleotide with 5 bp spacing showed much less evidence of enhanced stability of the [2x (dimeric)] complex, suggesting that protein:protein interaction was less likely when the DNA spacer was of inadequate length (Figure 7B). □

#### Discussion:

- 30 The production of three purified Stat1 protein preparations from recombinant DNA constructs was achieved: Stat1 $\alpha$  and Stat1 $\beta$  from baculovirus infected insect cells, and a Stat1tc from *E. coli*. Digestion of purified Stat1 $\alpha$  protein suggested a compact domain in the amino terminus of 131 amino acids and a relatively protease-resistant large carboxyl terminal fragment (132-712). Activated EGF-receptor partially purified from membranes

by immunoprecipitation was capable of *in vitro* catalysis of the phosphorylation of tyrosine 701, of Stat1 $\alpha$ , Stat1 $\beta$ , and Stat1 $\gamma$ . This is the same tyrosine that is phosphorylated *in vivo* by either IFN- $\alpha$ , IFN- $\gamma$  or EGF treatment of cells (Shuai *et al.*, 1992; Shuai *et al.*, 1993a). This *in vitro* approach was more efficient in generating activated Stat1 molecules than previous attempts that employed either co-infection of Stat1 and a JAK kinase in the baculovirus/insect cell system *in vivo*, or *in vitro* kinase assays with JAK kinases [unpublished observations and (Yan H. *et al.*, 1996)]. These results on *in vitro* phosphorylation of the protein plus alkylation to prevent aggregation, coupled with an adequate chromatographic protocol, allowed the purification of milligram quantities of activated protein. These techniques are also applicable to other Stat molecules such as Stat 2, 3, 4, 5A, 5B, and 6.

Analysis of the peptides derived from the purified phosphorylated protein by mass spectrometry, did not reveal significant contamination with unreacted Stat monomers. All three tyrosine-phosphorylated Stat1 derivatives dimerized and, as tested by EMSA, bound to the same DNA oligonucleotides previously shown to bind activated Stat1 in cell extracts.

The structure of the Stat protein is expected to be complex considering the number of interactions these proteins must undergo. The region from residues 400-500 specifies DNA contacts (Horvath *et al.*, 1995), while the carboxyl terminal half of the molecule contains the recognizable SH2 and putative SH3 domains (Fu, 1992; Schindler *et al.*, 1992), and the carboxyl terminus comprises the transactivation domain (Muller *et al.*, 1993; Wen *et al.*, 1995). From the digestion by proteases which released an amino terminal and a carboxyl terminal fragment a compact structure for the amino terminal of about 131 amino acids, is indicated. In addition there is a large stable fragment beginning at amino acid 132 that can be phosphorylated on a specific tyrosine and dimerize. The Stat1 protein binds to various DNA fragments with a  $K_{eq}$  of  $1 \times 10^{-9}$  M. Compared to other regulatory proteins this is a relatively modest affinity. Despite having similar apparent  $K_{eq}$  values, the binding with DNA may differ significantly in rates of association with and dissociation from the Stat protein. The Stat1 protein achieves equilibrium in DNA binding very rapidly, far quicker (less than 30 seconds) than the EMSA technique can determine. When the stability of Stat1 protein preparations to the various Stat1 binding sites were examined, measurable differences became apparent. Although the

protein/DNA complex had a half life of no more than 3 minutes for any of the sites tested, the "off" times for different oligonucleotides varied by at least six-fold. The difference between "strong" and "weak" oligonucleotide binding as detected in gel shift assays was found to be due to the rapid "off" time in competition assays with the displacement from "weak" sites being essentially instantaneous. Regarding the DNA binding activities of the Stat dimer to a single recognition sequence, no differences between the full length Stat1 $\alpha$  and the carboxyl- and amino terminally truncated Stat1tc was observed.

The new finding of great potential biological relevance in these studies concerns the cooperative stabilization of Stat homodimers on neighboring binding sites. This was observed when two tandem sites (separated by 10 or 15 bp) were both occupied by homodimers. A large complex was formed consisting presumably of two homodimers which was more stable to competition with unlabelled oligonucleotides than one dimer binding to a single site. This interaction required a minimum spacing (greater than 5 basepairs) between adjacent sites and was strongly orientation-dependent, i.e., it occurred only if both recognition sequences were in tandem.

Additionally a domain in the Stat1 molecule required for this dimer:dimer interaction was determined. The Stat1 $\beta$  lacking the carboxyl terminal 38 amino acids showed the same stabilization of the [2x (dimeric)] Stat complex on the DNA as the full length protein. However, the truncated protein Stat1tc that lacks the amino terminal 131 amino acids (as well as the carboxyl terminal sequence) formed the higher order complex less well, and this complex was not stabilized during oligonucleotide competition. Thus the amino terminal 131 amino acids of Stat1 defined by proteolysis as a stable domain, and which is dispensable for dimer formation and binding to single DNA sites, participates in Stat dimer:dimer interaction on tandem DNA sites. Interestingly, the isolated amino terminal domain dimerizes in solution. The amino terminus of the Stats shows rather high sequence homology (Schindler and Darnell, 1995), indicating that protein:protein interaction in this domain is of general importance in Stat function. Since there is evidence from the mig-gene (Guyer *et al.*, 1995) that neighboring "weak" Stat binding sites are required for a IFN- $\gamma$  response, it indicates that the interaction we describe has a biological role.

The present invention is not to be limited in scope by the specific embodiments described

herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

5

It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description.

- 10 Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

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The citation of any reference herein should not be construed as an admission that such reference is available as "Prior Art" to the instant application.

WHAT IS CLAIMED IS:

- 1 1. A protein having an amino acid sequence that is substantially similar to that of  
2 SEQ ID NO:3.
- 1 2. The protein of Claim 1 having the amino acid sequence of SEQ ID NO:3.
- 1 3. The protein of Claim 1 purified to exhibit a single protein band on 7% SDS-  
2 PAGE.
- 1 4. The protein of Claim 1 comprising a converted cysteine.
- 1 5. The protein of Claim 4 wherein the converted cysteine is an alkylated cysteine.
- 1 6. The protein of Claim 4 wherein the converted cysteine is a cysteine substituted  
2 with an alternative polar neutral amino acid.
- 1 7. The protein of Claim 6 wherein the alternative polar neutral amino acid is selected  
2 from the group consisting of a glycine, a serine, and a threonine.
- 1 8. The protein of Claim 4 comprising three converted cysteines, wherein the  
2 converted cysteines are Cysteine 155, Cysteine 440, and Cysteine 492.
- 1 9. The protein of Claim 8 wherein the three converted cysteines are alkylated  
2 cysteines.
- 1 10. The protein of Claim 9 comprising a phosphorylated tyrosine at tyrosine 701.
- 1 11. The protein of Claim 10 purified to exhibit a single protein band on 7% SDS-  
2 PAGE.
- 1 12. A purified N-terminal peptide fragment of a Stat protein having an amino acid  
2 sequence substantially similar to SEQ ID NO:4.

1 13. The purified N-terminal peptide fragment of Claim 12 having an amino acid  
2 sequence of SEQ ID NO:4.

1 14. A nucleic acid that comprises a nucleotide sequence that codes for the expression  
2 of a truncated Stat protein having an amino acid sequence that is substantially similar to  
3 that of SEQ ID NO:3.

1 15. The nucleic acid of Claim 14 wherein the nucleotide sequence is SEQ ID NO:5.

1 16. A nucleic acid comprising a nucleotide sequence encoding the N-terminal peptide  
2 fragment of a Stat protein having an amino acid sequence substantially similar to SEQ ID  
3 NO:4.

1 17. The nucleic acid of Claim 16 wherein the nucleotide sequence is SEQ ID NO:6.

1 18. A method of separating the phosphorylated form of a protein from the  
2 nonphosphorylated form comprising:  
3 (a) placing a mixture of the phosphorylated form of a protein and the  
4 nonphosphorylated form of the protein onto heparin agarose; wherein the phosphorylated  
5 form of the protein and the nonphosphorylated form of the protein bind to heparin  
6 agarose;

7 (b) eluting the phosphorylated form of the protein and the nonphosphorylated  
8 form of the protein from the heparin agarose as a function of salt concentration;  
9 wherein the nonphosphorylated form of the protein elutes prior to the  
10 phosphorylated form of the protein; and

11 wherein the protein is selected from the group consisting of a Stat protein and a  
12 truncated Stat protein.

1 19. The method of Claim 18 wherein eluting the heparin agarose as a function of salt  
2 concentration is performed with a salt gradient.

1 20. The method of Claim 18 wherein the protein is a Stat protein having an amino  
2 acid sequence selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2.

1 21. The method of Claim 18 wherein the protein is a truncated Stat protein having an  
2 amino acid sequence substantially similar to SEQ ID NO:3.

1 22. The method of Claim 18 wherein eluting the heparin agarose as a function of salt  
2 concentration is performed stepwise with an approximately 0.15 M monovalent salt elution  
3 step, followed by an approximately 0.4 M monovalent salt elution step; and wherein the  
4 unphosphorylated form of the protein elutes with the first step and the phosphorylated  
5 form of the protein elutes with the second step.

1 23. The method of Claim 22 wherein the protein has a converted cysteine.

1 24. The method of Claim 23 wherein the protein is a Stat protein having an amino  
2 acid sequence selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2.

1 25. The method of Claim 23 wherein the protein is a truncated Stat protein having an  
2 amino acid sequence that is substantially similar to SEQ ID NO:3.

1 26. A method of preparing a purified alkylated Stat protein comprising:  
2 (a) placing an expression vector containing a nucleic acid encoding a Stat  
3 protein into a compatible host cell, wherein the Stat protein is expressed;  
4 (b) growing the compatible host cell;  
5 (c) releasing the expressed Stat protein from the host cell;  
6 (d) alkylating a cysteine of the expressed Stat protein, wherein the cysteine is  
7 involved in intersubunit aggregation, and wherein an alkylated Stat protein is formed; and  
8 (e) isolating the alkylated Stat protein; wherein said isolating yields a purified  
9 alkylated Stat protein.

1 27. The method of Claim 26 wherein the Stat protein has an amino acid sequence  
2 selected from the group consisting of SEQ ID NO:1, and SEQ ID NO:2.

1 28. The method of Claim 27 further comprising the step of phosphorylating the  
2 alkylated Stat protein.

1 29. The method of Claim 27 wherein said alkylating is performed by incubating the

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## 2 Stat protein with N-ethyl maleimide.

1       30.     A method of preparing a purified alkylated truncated Stat protein comprising:

2               (a)     placing an expression vector containing a nucleic acid encoding a truncated

3     Stat protein into a compatible host cell, wherein the truncated Stat protein is expressed;

4               (b)     growing the compatible host cell;

5               (c)     releasing the expressed truncated Stat protein from the host cell;

6               (d)     alkylating a cysteine of the expressed truncated Stat protein, wherein the

7     cysteine is involved in intersubunit aggregation, and wherein an alkylated truncated Stat

8     protein is formed; and

9               (e)     isolating the alkylated truncated Stat protein; wherein said isolating yields

10    a purified alkylated truncated Stat protein;

11               wherein the truncated Stat protein has an N-terminal sequence that is substantially

12    similar to the N-terminus of the corresponding Stat protein following the cleavage of the

13    proteolytic sensitive N-terminal domain from the corresponding Stat protein; and

14               wherein the carboxyl terminus of the truncated Stat protein extends at least to the

15    phosphorylatable tyrosine required for homodimerization.

1    31.    The method of Claim 30 wherein about 40 to 50 mg of purified alkylated  
2    truncated Stat protein can be obtained from 6 liters of starting culture.

1    32.    The method of Claim 30 further comprising the step of phosphorylating the  
2    alkylated truncated Stat protein.

1 33. The method of Claim 32 wherein the truncated Stat protein has an amino acid  
2 sequence that is substantially similar to SEQ ID NO:3.

1        34.     A method of preparing a purified substituted Stat protein comprising:

2                (a)     placing an expression vector into a compatible host cell, wherein the

3     expression vector contains a nucleic acid encoding a substituted Stat protein that has an

4     alternative polar neutral amino acid substituted for a cysteine of the Stat protein, wherein

5     the cysteine is involved in intersubunit aggregation, and wherein the substituted Stat

6     protein is expressed;

7                (b)     growing the compatible host cell;

8           (c)     releasing the expressed substituted Stat protein from the host cell; and  
9           (d)     isolating the substituted Stat protein; wherein said isolating yields the  
10      purified substituted Stat protein.

1    35.   The method of Claim 34 wherein the Stat protein has an amino acid sequence  
2    selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2.

1    36.   The method of Claim 34 further comprising the step of phosphorylating the  
2    substituted Stat protein.

1    37.   The method of Claim 34 wherein the alternative polar neutral amino acid is  
2    selected from the group consisting of a glycine, a serine, and a threonine.

1    38.   A method of preparing a purified substituted truncated Stat protein comprising:  
2        (a)     placing an expression vector into a compatible host cell, wherein the  
3     expression vector contains a nucleic acid encoding a substituted truncated Stat protein that  
4     has an alternative polar neutral amino acid substituted for a cysteine of the truncated Stat  
5     protein, wherein the cysteine is involved in intersubunit aggregation, and wherein the  
6     substituted truncated Stat protein is expressed;

7        (b)     growing the compatible host cell;  
8        (c)     releasing the expressed substituted truncated Stat protein from the host  
9     cell; and

10       (d)     isolating the substituted truncated Stat protein; wherein said isolating  
11    yields the purified substituted Stat protein;

12       wherein the truncated Stat protein has an N-terminal sequence that is substantially  
13    similar to the N-terminus of the corresponding Stat protein following the cleavage of the  
14    proteolytic sensitive N-terminal domain from the corresponding Stat protein; and

15       wherein the carboxyl terminus of the truncated Stat protein extends at least to the  
16    phosphorylatable tyrosine required for dimerization.

1    39.   The method of Claim 38 wherein about 40 to 50 mg of purified substituted Stat  
2    protein can be obtained from 6 liters of starting culture.

1    40.   The method of Claim 38 further comprising the step of phosphorylating the

2 substituted truncated Stat protein.

1 41. The method of Claim 40 wherein the truncated Stat protein has an amino acid  
2 sequence that is substantially similar to SEQ ID NO:3.

1 42. A method for identifying a drug that enhances or diminishes the ability of STAT  
2 protein dimers to induce the expression of a gene operably under the control of a promoter  
3 containing at least two adjacent weak binding sites for STAT protein dimers comprising:

4 (a) measuring the level of expression of a first reporter gene and a second  
5 reporter gene contained by a host cell in the presence and absence of a prospective drug;  
6 wherein the first reporter gene is operably linked to a first promoter containing at least  
7 two adjacent weak binding sites for STAT protein dimers, and the second reporter gene is  
8 operably linked to a second promoter comprising at least one strong binding site for a  
9 STAT protein dimer; wherein the binding of STAT protein dimers to the two adjacent  
10 weak binding sites induces the expression of the first reporter gene, and wherein the  
11 binding of the STAT protein dimer to the strong binding site induces the expression of the  
12 second reporter gene; and wherein the host cell contains STAT protein dimers;

13 (b) comparing the level of expression of the first reporter gene with that of the  
14 second reporter gene in the presence and absence of the prospective drug, wherein when  
15 the presence of the prospective drug results in an increase in the level of expression of the  
16 first reporter gene but not that of the second reporter gene, the prospective drug is  
17 identified as a drug that enhances the ability of STAT protein dimers to induce the  
18 expression of a gene operably under the control of a promoter containing at least two  
19 adjacent weak binding sites for STAT protein dimers; and when the presence of a  
20 prospective drug results in a decrease in the level of expression of the first reporter gene  
21 but not that of the second reporter gene the prospective drug is identified as a drug that  
22 inhibits the ability of STAT protein dimers to induce the expression of a gene operably  
23 under the control of a promoter containing at least two adjacent weak binding sites for  
24 STAT protein dimers.

1 43. The method of Claim 42 wherein the host cell is a mammalian cell.

1 44. The method of Claim 42 wherein the first reporter gene is contained by a first host  
2 cell, and the second reporter gene is contained by a second host cell; and wherein the first

3 host cell and second host cell both contain STAT protein dimers.

1 45. The method of Claim 42 wherein the weak STAT binding sites are selected from  
2 the group consisting of sites present in the regulatory regions of the MIG gene, the c-fos  
3 gene and the interferon- $\gamma$  gene.

1 46. A method for identifying a drug that modulates the ability of adjacent STAT  
2 protein dimers to interact and bind to adjacent DNA binding sites comprising:

3           a) measuring the level of expression of a reporter gene in a first host cell in  
4 the presence and absence of a test compound, wherein the first host cell contains a  
5 reporter gene operably linked to a promoter comprising at least two adjacent weak binding  
6 sites for the STAT protein dimer, such that binding of the dimers to the promoter causes  
7 expression of the reporter gene;

8           b) measuring the level of expression of a reporter gene in a second host cell  
9 in the presence and absence of the test compound, wherein the second host cell contains a  
10 reporter gene operably linked to a second promoter comprising at least one strong binding  
11 site for the STAT protein dimer, such that binding of the dimer to the promoter causes  
12 expression of the reporter gene; and

13 c) comparing the level of expression of the reporter gene in the first host cell  
14 in the presence and absence of the test compound with the level of expression of the  
15 reporter gene in the second host cell in the presence and absence of the test compound,  
16 wherein a test compound which causes an increase in the level of expression of the  
17 reporter gene in said first host cell but not in said second host cell is identified as a drug  
18 that enhances the interaction between adjacent STAT protein dimers, and a test compound  
19 which causes a decrease in the level of expression of the reporter gene in the first host cell  
20 but not in the second host cell is identified as a drug that inhibits the interaction between  
21 adjacent activated STAT dimers.

1 47. The method of Claim 46 wherein the first host cell and second host cell are  
2 mammalian cells.

1 48. The method of Claim 46 wherein the weak STAT binding sites are selected from  
2 the group consisting of sites present in the regulatory regions of the MIG gene, the c-fos  
3 gene and the interferon- $\gamma$  gene.

1    49.    A method for identifying a drug that modulates the ability of adjacent STAT  
2    protein dimers to interact and bind to adjacent DNA binding sites comprising:  
3         a)      measuring the binding affinity of the STAT protein, or a fragment thereof  
4    that comprises the N-terminal domain, to a nucleic acid comprising 2 adjacent weak STAT  
5    DNA binding sites in the presence and absence of a test compound;  
6         b)      measuring the binding affinity of the STAT protein, or the fragment, to a  
7    nucleic acid comprising a single strong STAT binding site in the presence and absence of  
8    the test compound: and  
9         c)      comparing the binding affinity measured in step (a) in the presence and  
10   absence of the test compound with the binding affinity measured in step (b) in the  
11   presence and absence of the test compound, wherein a test compound which causes an  
12   increase in the binding affinity measured in step (a) but not in the binding affinity  
13   measured in step (b) is identified as a drug that enhances the interaction between adjacent  
14   activated STAT dimers, and a test compound which causes a decrease in the binding  
15   affinity measured in step (a) but not in the binding affinity measured in step (b) is  
16   identified as a drug that inhibits the interaction between adjacent activated STAT dimers.

1    50.    A method for identifying a drug that modulates the ability of adjacent STAT  
2    protein dimers to interact and bind to adjacent DNA binding sites comprising:  
3         a)      measuring the binding affinity of the STAT protein, or a fragment thereof  
4    comprising the N-terminal domain, to a nucleic acid comprising 2 adjacent weak STAT  
5    DNA binding sites in the presence and absence of a test compound;  
6         b)      measuring the binding affinity of a truncated form of the STAT protein  
7    lacking the N-terminal domain with the nucleic acid in the presence and absence of the  
8   test compound; and  
9         c)      comparing the binding affinity measured in step (a) in the presence and  
10   absence of the test compound with the binding affinity measured in step (b) in the  
11   presence and absence of the test compound, wherein a test compound which causes an  
12   increase in the binding affinity measured in step (a) but not in the binding affinity  
13   measured in step (b) is identified as a drug that enhances the interaction between adjacent  
14   activated STAT dimers, and a test compound which causes a decrease in the binding  
15   affinity measured in step (a) but not in the binding affinity measured in step (b) is  
16   identified as a drug that inhibits the interaction between adjacent activated STAT dimers.

1 51. A method for identifying a drug that modulates the ability of adjacent STAT  
2 protein dimers to interact and bind to adjacent DNA binding sites comprising measuring  
3 the ability of a first preparation of a fragment of STAT protein dimer comprising the N-  
4 terminal domain to bind to a second preparation of a fragment of said STAT protein  
5 comprising the N-terminal domain in the presence and absence of a test compound,  
6 wherein a test compound which increases the ability of the first preparation to bind to the  
7 second preparation is identified as a drug that enhances the interaction between adjacent  
8 activated STAT dimers, and a test compound which decreases the ability of the first  
9 preparation to bind to the second preparation is identified as a drug that inhibits the  
10 interaction between adjacent activated STAT dimers.

1 52. The method of Claim 51 wherein either said first preparation or said second  
2 preparation is labeled.

1 53. The method of Claim 51 wherein said first preparation and said second preparation  
2 are labeled.

1 54. The method of claim 51 wherein said first preparation is bound to a solid support.

1 55. The method of Claim 54 wherein said second preparation is labeled.

ABSTRACT OF THE INVENTION

The present invention describes methods of producing milligram quantities of three forms of purified Stat1 protein from recombinant DNA constructs. In addition, the Stat proteins may be isolated in their phosphorylated or nonphosphorylated forms (Tyr 701). The proteins can be produced in baculovirus infected insect cells, or *E. coli*. A compact domain in the amino terminus of Stat1 $\alpha$  was isolated and found to enhance DNA binding due to its ability to interact with a neighboring Stat protein. A relatively protease-resistant recombinant truncated form of the Stat protein was isolated in 40-50 mg quantities. Purification of the Stat proteins were performed after modifying specific cysteine residues of the Stat proteins to prevent aggregation. Activated EGF-receptor partially purified from membranes by immunoprecipitation was shown to be capable of *in vitro* catalysis of the phosphorylation of the tyrosine residue of Stat1 known to be phosphorylated *in vivo*. Techniques are enclosed to separate the phosphorylated from the nonphosphorylated Stat proteins. The techniques disclosed are general for Stat proteins and may be used to isolate large quantities of purified Stat 2, 3, 4, 5A, 5B and 6. Methods for using purified Stat proteins, truncated Stat proteins, or Stat N-terminal fragments for drug discovery are also disclosed.

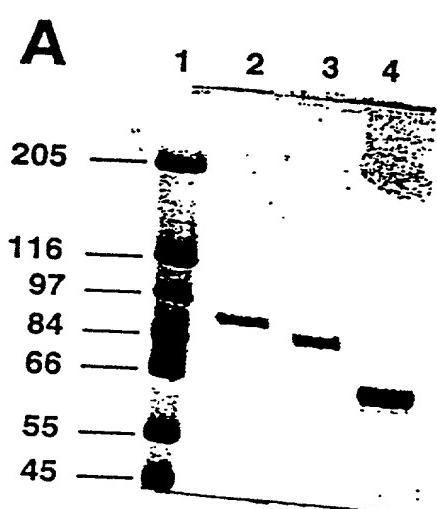


Figure 1A

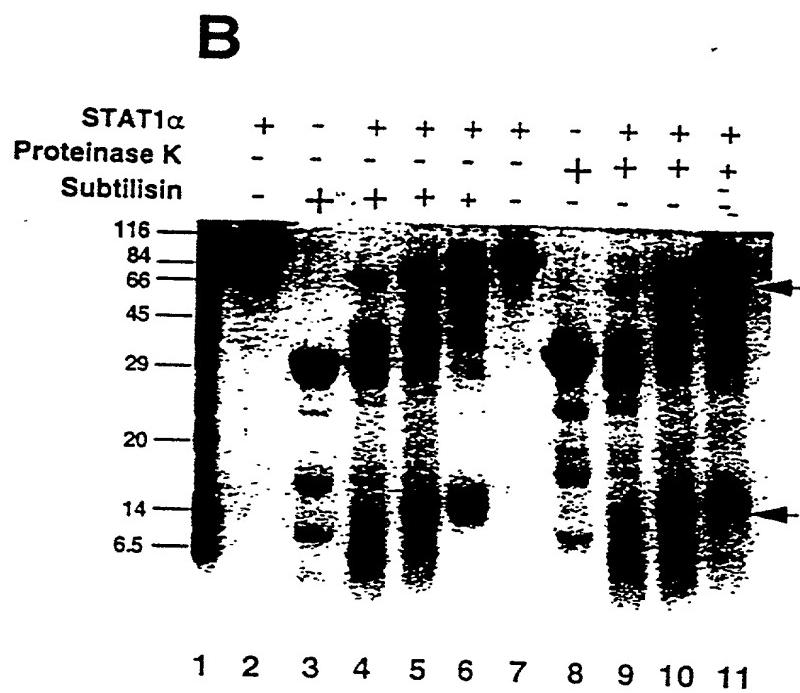


Figure 1B

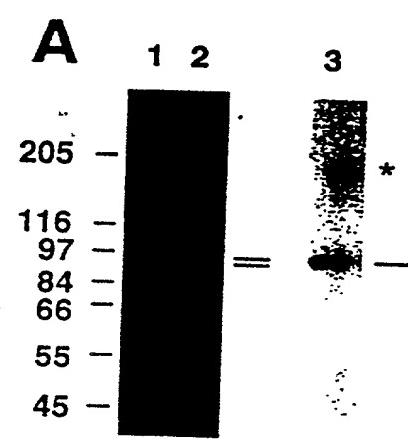


Figure 2A

600-1-182 N (Sheet 4 of 14)

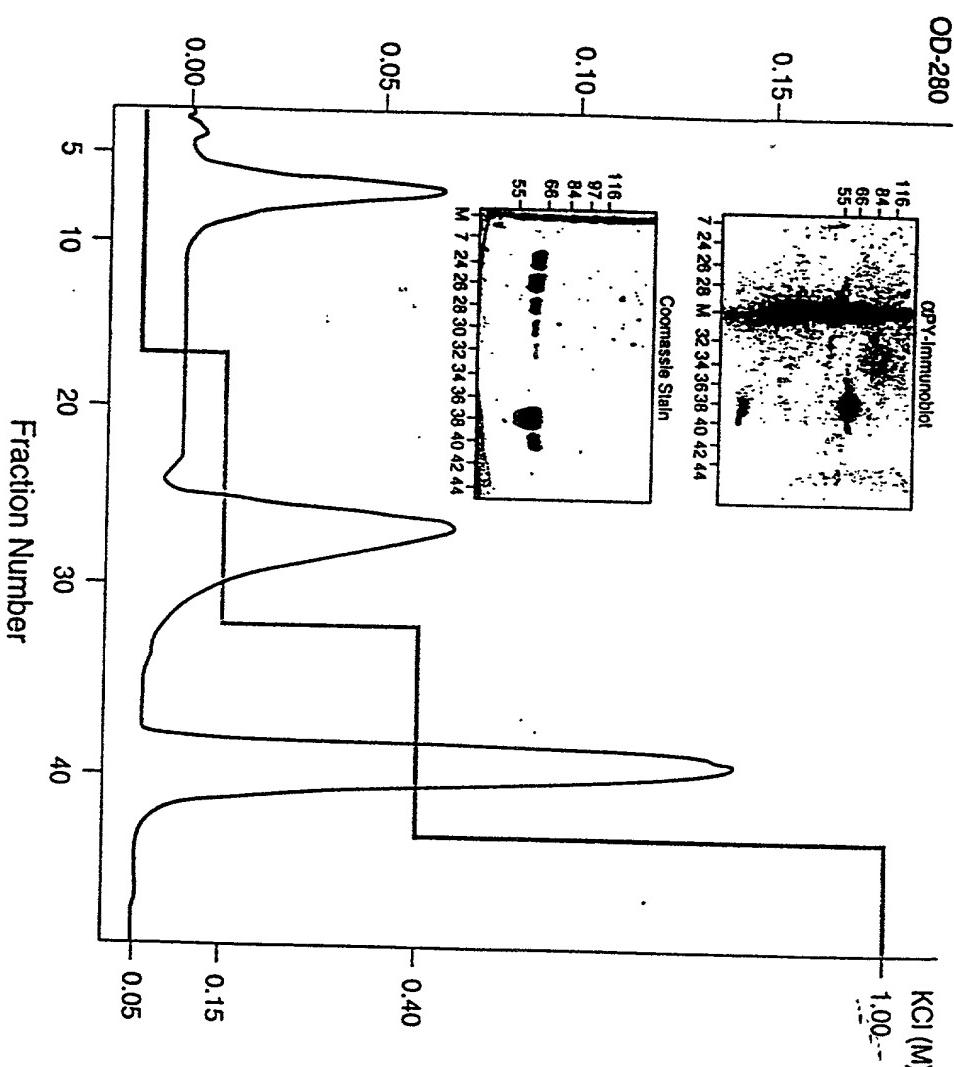
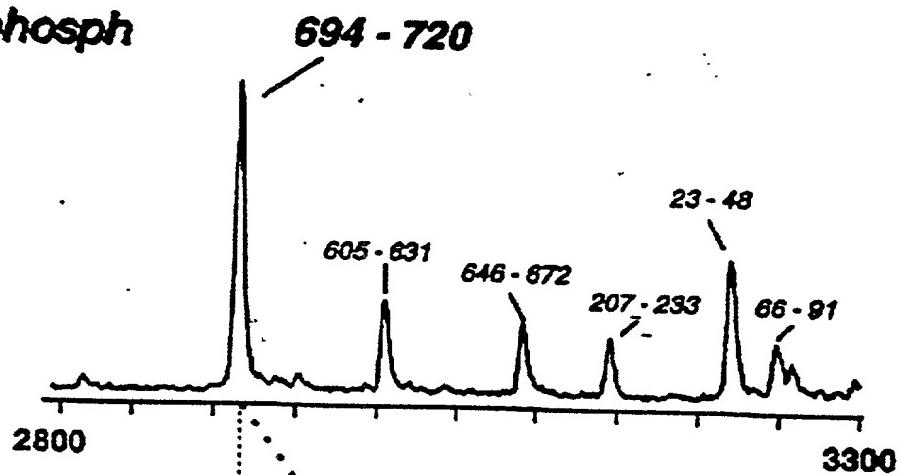


Figure 2B

**C**

(-) phosph



(+)-phosph

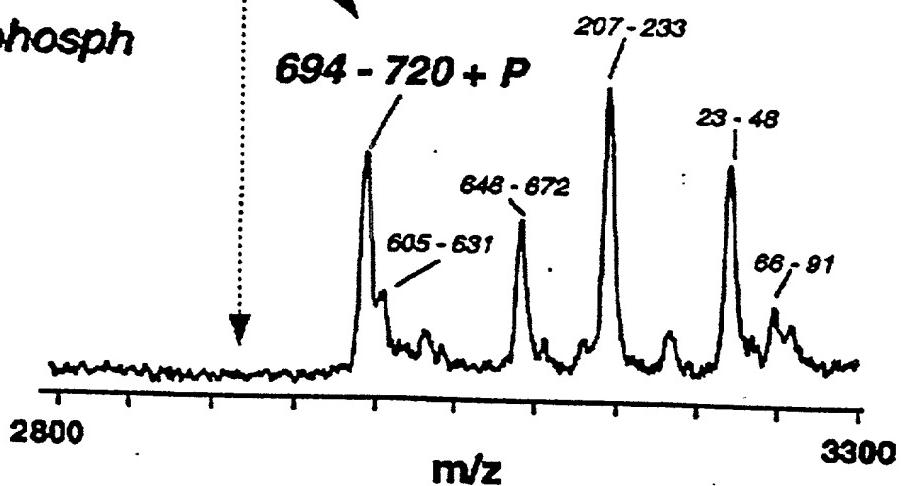


Figure 2C

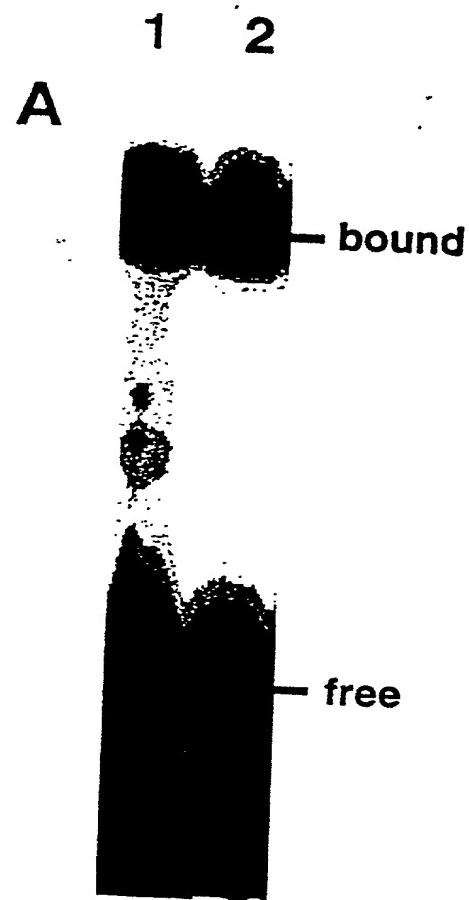


Figure 3A

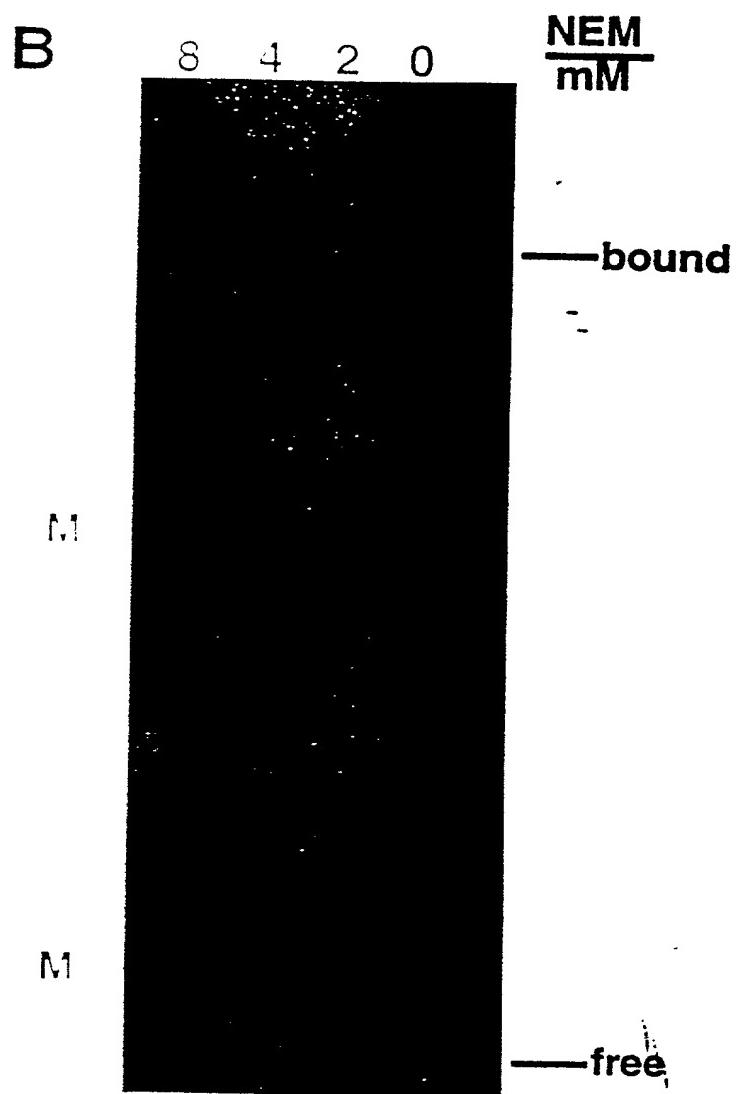


Figure 3B

600-1-182 N (Sheet 8 of 14)

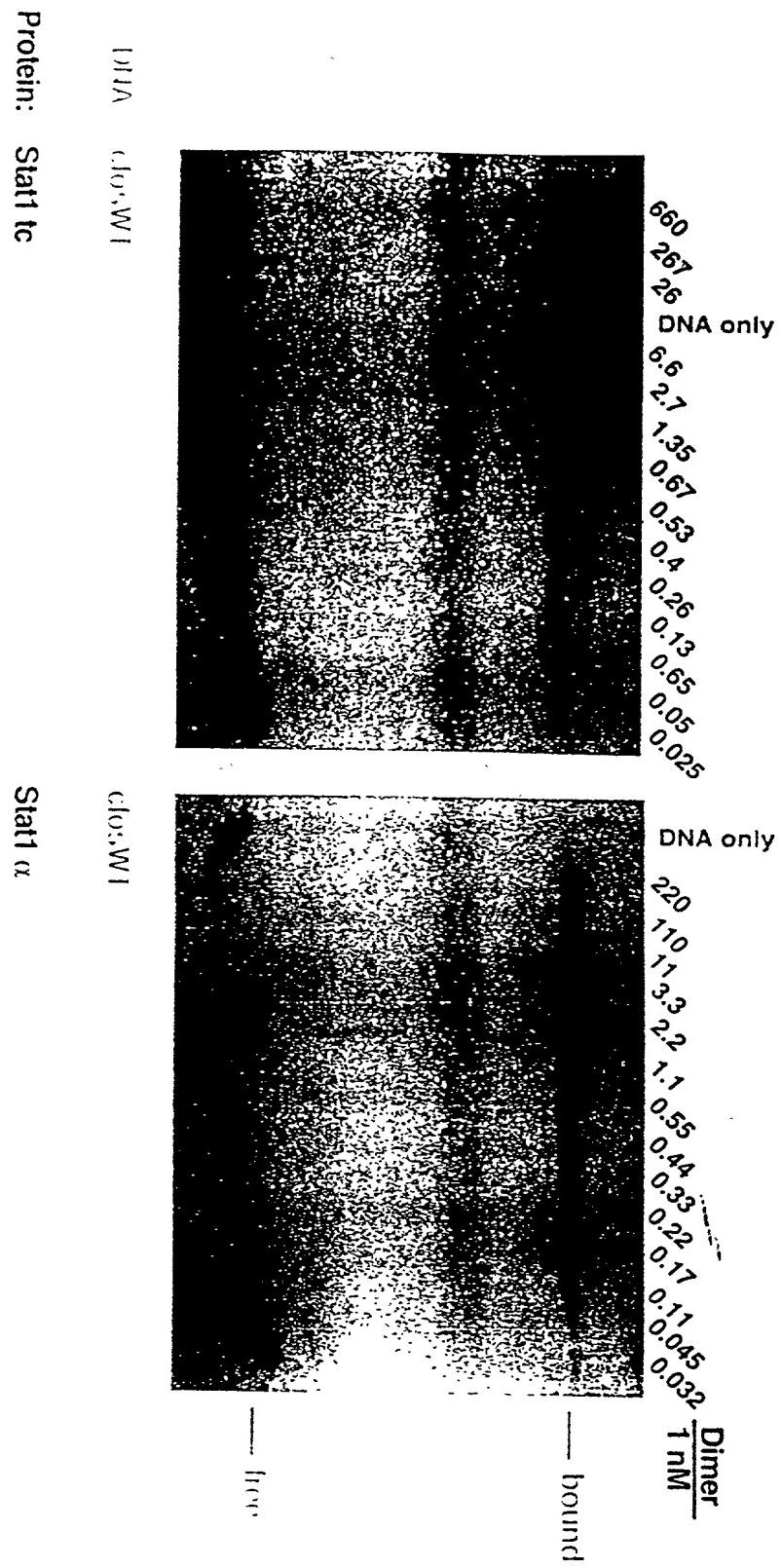


Figure 4  
0.9431605 1.10299

600-1-182 N (Sheet 9 of 14)

A

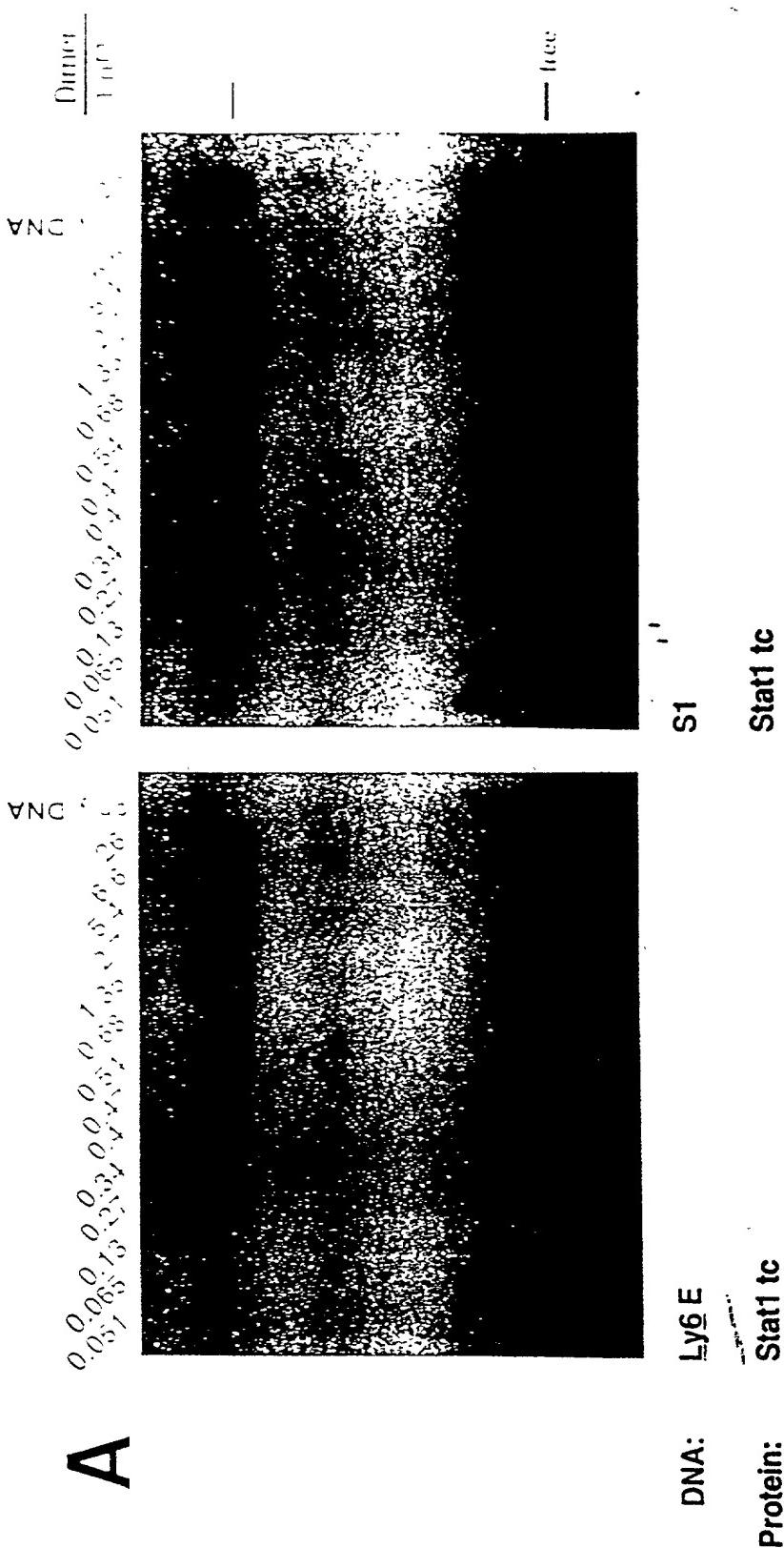


Figure 5A

600-1-182 N (Sheet 10 of 14)

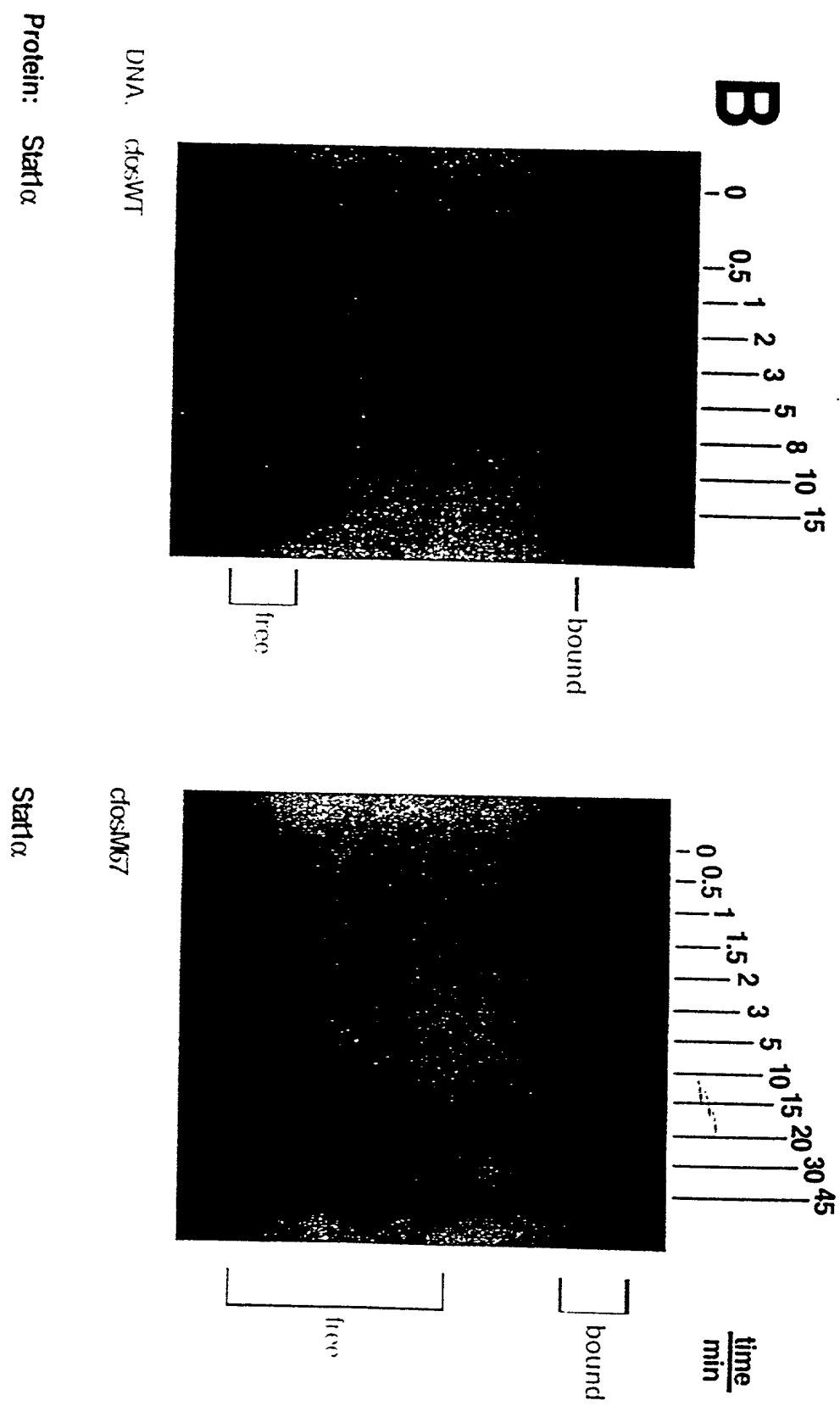
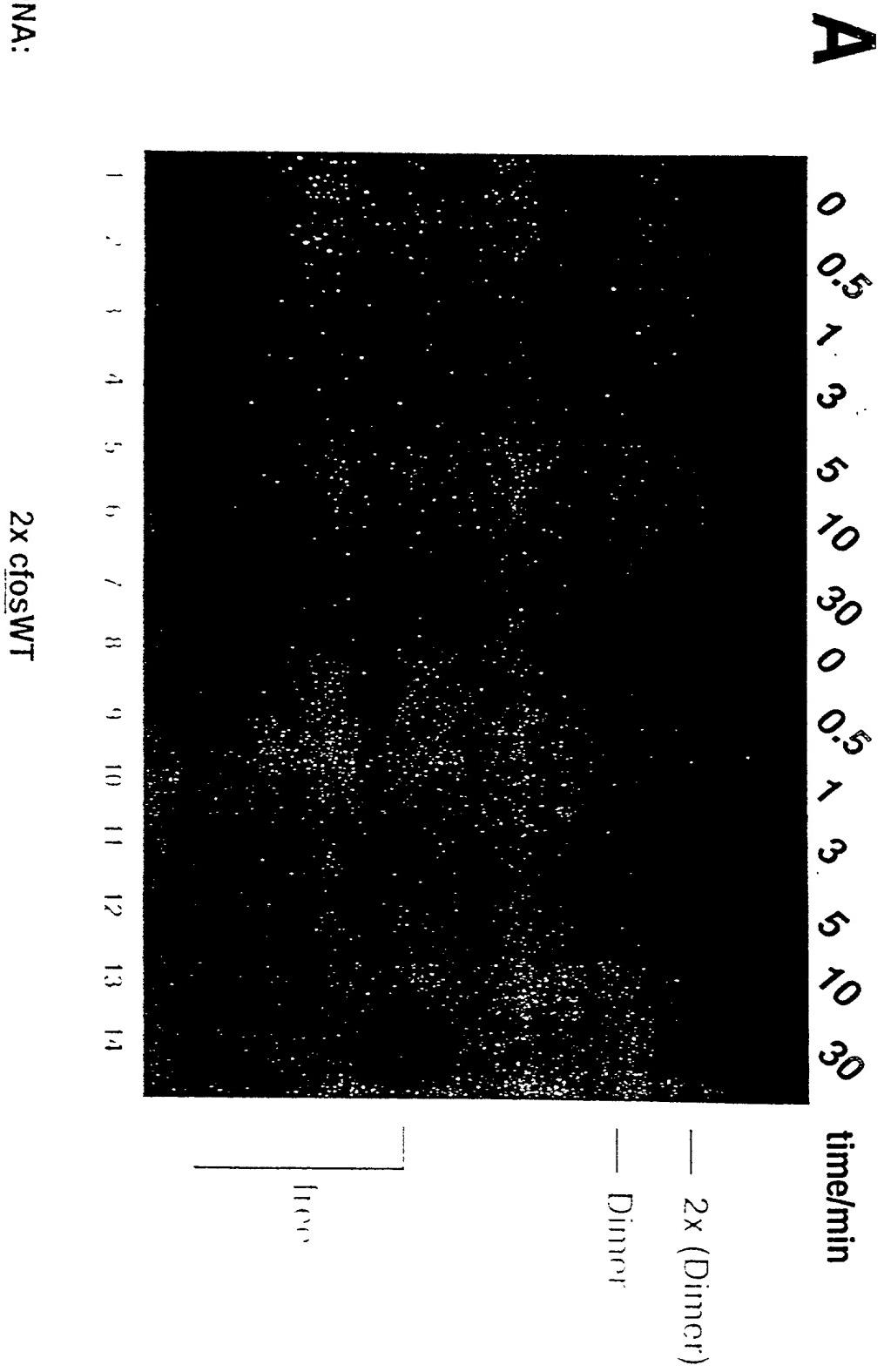


Figure 5B



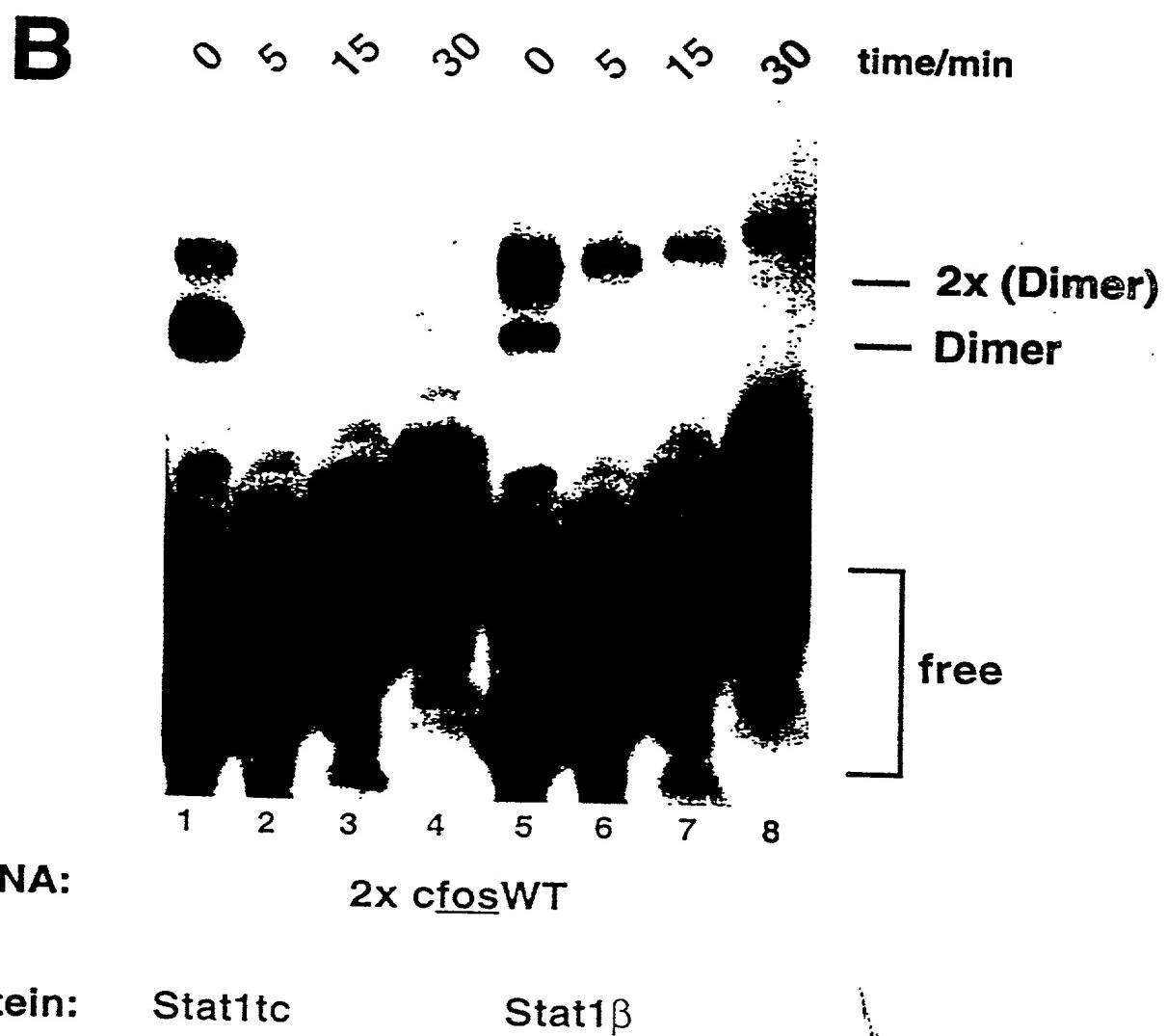


Figure 6B

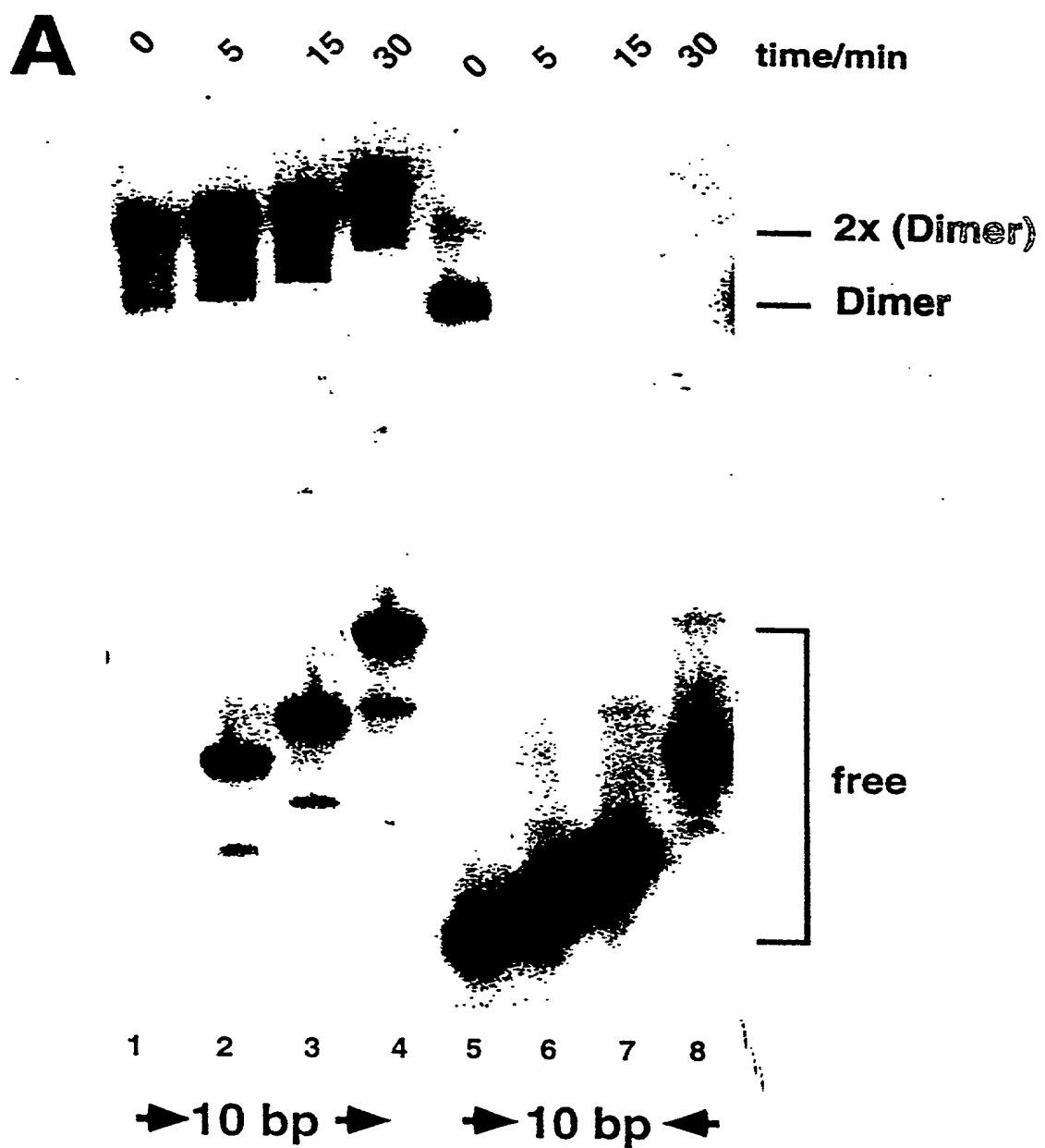


Figure 7A

600-1-182 N (Sheet 14 of 14)

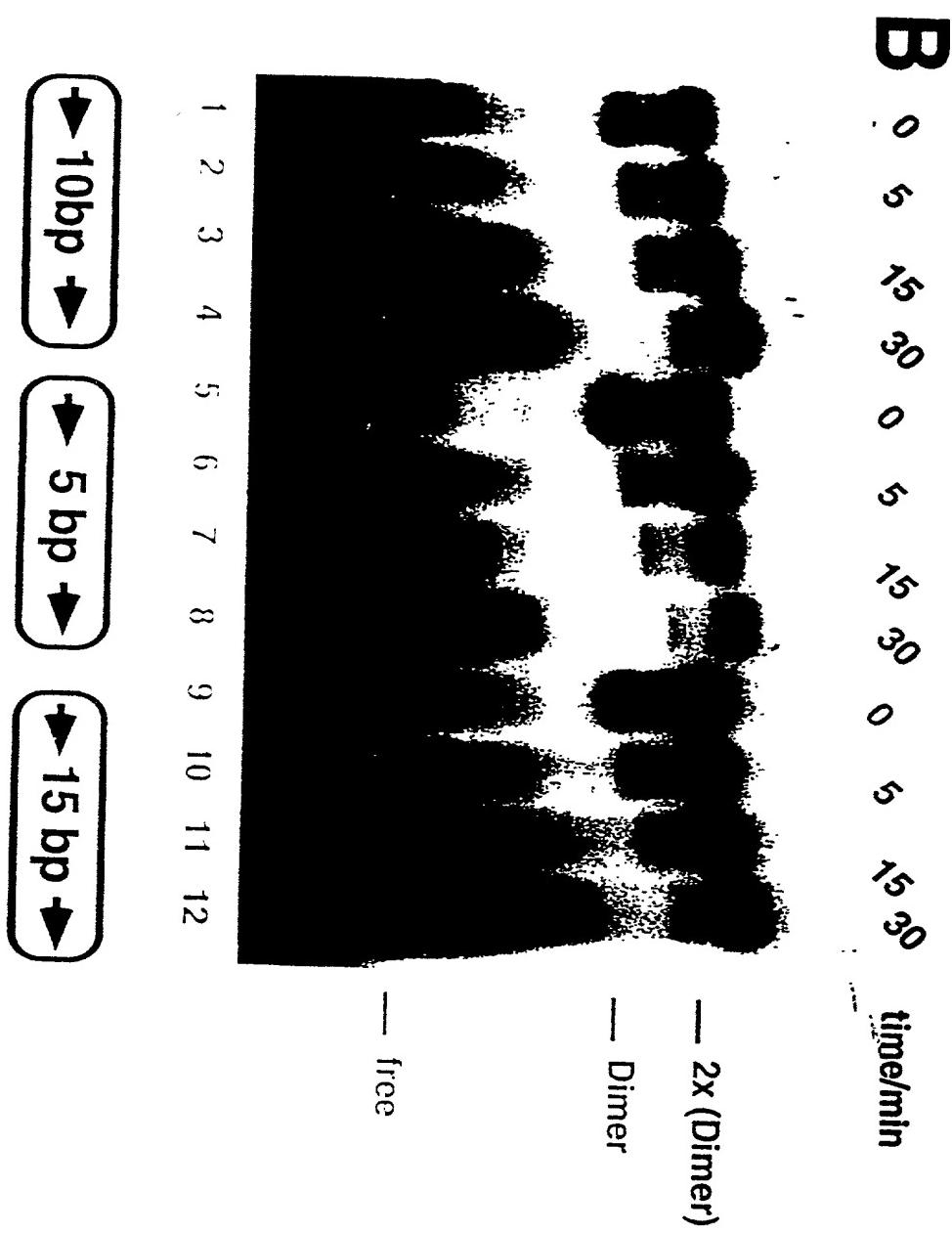


Figure 7B

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**DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION**

As below named inventors, we hereby declare that:

Our residence, post office address and citizenship are as stated below under our names.

We believe that we are the original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled

**PURIFIED STAT PROTEINS AND METHODS OF PURIFYING THEREOF**

the Specification of which

- [ ] is attached hereto  
[X] was filed on October 15, 1997  
as Application Serial No. 08/951,130  
and was amended on \_\_\_\_\_ (if applicable).

We hereby state that we have reviewed and understand the contents of the above-identified Specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, 1.56(a).

We hereby claim foreign priority benefits under Title 35, United States Code, §119 of any provisional application filed in the United States in accordance with 35 U.S.C. §1.119(e), or any application for patent that has been converted to a Provisional Application within one (1) year of its filing date, or any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

<u>PRIOR FILED APPLICATION(S)</u>			
<u>APPLICATION NUMBER</u>	<u>COUNTRY</u>	<u>(DAY/MONTH/YEAR FILED)</u>	<u>PRIORITY CLAIMED</u>
60/028,176	United States	15 October 1996	Yes

We hereby claim the benefit under Title 35, United States Code, §120 of any United States application listed below, and, insofar as the subject matter of each of the claims of this application is not disclosed in any prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a), which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION NO.	FILING DATE <u>(DAY/MONTH/YEAR)</u>	STATUS - PATENTED, PENDING, <u>ABANDONED</u>
None		

We hereby appoint as our attorneys or agents the following persons: Jack Matalon, (Attorney, Registration No. 22,441); Stefan J. Klauber (Attorney, Registration No. 22,604); David A. Jackson (Attorney, Registration No. 26,742); Michael D. Davis (Attorney, Registration No. 39,161); Joseph M. Homa (Attorney, Registration No. 40,023); and Christine E. Dietzel (Agent, Registration No. 37,309), said attorneys or agents with full power of substitution and revocation to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Please address all correspondence regarding this application to:

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411 HACKENSACK AVENUE  
HACKENSACK, NEW JERSEY 07601

Direct all telephone calls to David A. Jackson at (201) 487-5800.

We hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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SIGNATURE OF INVENTOR Uwe Vinkemeier

DATE 2/5/93

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SIGNATURE OF INVENTOR James E. Darnell, Jr.

DATE 2/9/98

**DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION**

As below named inventors, we hereby declare that:

Our residence, post office address and citizenship are as stated below under our names.

We believe that we are the original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled

**PURIFIED STAT PROTEINS AND METHODS OF PURIFYING THEREOF**

the Specification of which

[X] is attached hereto  
[ ] was filed on \_\_\_\_\_  
as Application Serial No. \_\_\_\_\_  
and was amended on \_\_\_\_\_ (if applicable).

We hereby state that we have reviewed and understand the contents of the above-identified Specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, 1.56(a).

We hereby claim foreign priority benefits under Title 35, United States Code, §119 of any provisional application filed in the United States in accordance with 35 U.S.C. §1.119(e), or any application for patent that has been converted to a Provisional Application within one (1) year of its filing date, or any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

<u>PRIOR FILED APPLICATION(S)</u>			
<u>APPLICATION NUMBER</u>	<u>COUNTRY</u>	<u>(DAY/MONTH/YEAR FILED)</u>	<u>PRIORITY CLAIMED</u>
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<u>APPLICATION NO.</u>	<u>FILING DATE (DAY/MONTH/YEAR)</u>	<u>STATUS - PATENTED, PENDING, ABANDONED</u>
None		

We hereby appoint as our attorneys or agents the following persons: Jack Matalon, (Attorney, Registration No. 22,441); Stefan J. Klauber (Attorney, Registration No. 22,604); David A. Jackson (Attorney, Registration No. 26,742); Barbara L. Renda (Attorney, Registration No. 27,626); Michael D. Davis (Attorney, Registration No. 39,161); and Joseph M. Homa (Attorney, Registration No. 40,023), said attorneys or agents with full power of substitution and revocation to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Please address all correspondence regarding this application to:

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HACKENSACK, NEW JERSEY 07601

Direct all telephone calls to David A. Jackson at (201) 487-5800.

We hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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DATE \_\_\_\_\_

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SIGNATURE OF INVENTOR \_\_\_\_\_

DATE \_\_\_\_\_

DRAFTING DRAWING

SEQUENCE LISTING

<110> Vinkemeier, Uwe  
Darnell Jr., James E.

<120> PURIFIED STAT PROTEINS AND METHODS OF PURIFYING THEREOF

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100 105 110  
  
Arg Lys Ile Leu Glu Asn Ala Gln Arg Phe Asn Gln Ala Gln Ser Gly  
115 120 125

DRAFT - DO NOT CITE

Asn Ile Gln Ser Thr Val Met Leu Asp Lys Gln Lys Glu Leu Asp Ser  
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Lys Val Arg Asn Val Lys Asp Lys Val Met Cys Ile Glu His Glu Ile  
145 150 155 160

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Glu Leu Thr Gln Asn Ala Leu Ile Asn Asp Glu Leu Val Glu Trp Lys  
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Asn Glu Arg Asn Thr Val Lys Gly Phe Arg Lys Phe Asn Ile Leu Gly  
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DRAFT

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420 425 430

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Glu Thr Thr Ser Leu Pro Val Val Val Ile Ser Asn Val Ser Gln Leu  
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Gly Leu Asn Val Asp Gln Leu Asn Met Leu Gly Glu Lys Leu Leu Gly  
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580 585 590

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Tyr Leu Ala Gln Trp Leu Glu Lys Gln Asp Trp Glu His Ala Ala Asn  
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Asp Val Ser Phe Ala Thr Ile Arg Phe His Asp Leu Leu Ser Gln Leu  
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Asp Asp Gln Tyr Ser Arg Phe Ser Leu Glu Asn Asn Phe Leu Leu Gln  
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Lys Arg Lys Glu Val Val His Lys Ile Ile Glu Leu Leu Asn Val Thr  
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Tyr Glu His Asp Pro Ile Thr Lys Asn Lys Gln Val Leu Trp Asp Arg  
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Arg Gln Pro Cys Met Pro Thr His Pro Gln Arg Pro Leu Val Leu Lys  
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Thr His Thr Lys Val Met Asn Met Glu Glu Ser Thr Asn Gly Ser Leu  
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Ala Ala Glu Phe Arg His Leu Gln Leu Lys Glu Gln Lys Asn Ala Gly  
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Thr Arg Thr Asn Glu Gly Pro Leu Ile Val Thr Glu Glu Leu His Ser  
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Leu Ser Phe Glu Thr Gln Leu Cys Gln Pro Gly Leu Val Ile Asp Leu  
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Glu Thr Thr Ser Leu Pro Val Val Val Ile Ser Asn Val Ser Gln Leu  
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Pro Ser Gly Trp Ala Ser Ile Leu Trp Tyr Asn Met Leu Val Ala Glu  
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Glu Asn Ile Asn Asp Lys Asn Phe Pro Phe Trp Leu Trp Ile Glu Ser  
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Asp Gln Gln Pro Gly Thr Phe Leu Leu Arg Phe Ser Glu Ser Ser Arg  
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35 40 45

Arg Glu His Glu Thr Asn Gly Val Ala Lys Ser Asp Gln Lys Gln Glu  
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Gln Leu Leu Leu Lys Lys Met Tyr Leu Met Leu Asp Asn Lys Arg Lys  
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Glu Val Val His Lys Ile Ile Glu Leu Leu Asn Val Thr Glu Leu Thr  
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Gln Asn Ala Leu Ile Asn Asp Glu Leu Val Glu Trp Lys Arg Arg Gln  
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Gln Ser Ala Cys Ile Gly Gly Pro Pro Asn Ala Cys Leu Asp Gln Leu  
115 120 125

Gln Asn Trp Phe Thr Ile Val Ala Glu Ser Leu Gln Gln Val Arg Gln  
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Gln Leu Lys Lys Leu Glu Glu Leu Glu Gln Lys Tyr Thr Tyr Glu His  
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Asp Pro Ile Thr Lys Asn Lys Gln Val Leu Trp Asp Arg Thr Phe Ser  
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195 200 205

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Lys Val Met Asn Met Glu Glu Ser Thr Asn Gly Ser Leu Ala Ala Glu  
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Val Asp Gln Leu Asn Met Leu Gly Glu Lys Leu Leu Gly Pro Asn Ala  
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Tyr Leu Ala Gln Trp Leu Glu Lys Gln Asp Trp Glu His Ala Ala Asn  
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Asp Val Ser Phe Ala Thr Ile Arg Phe His Asp Leu Leu Ser Gln Leu  
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Asp Asp Gln Tyr Ser Arg Phe Ser Leu Glu Asn Asn Phe Leu Leu Gln  
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His Asn Ile Arg Lys Ser Lys Arg Asn Leu Gln Asp Asn Phe Gln Glu  
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Asp Pro Ile Gln Met Ser Met Ile Ile Tyr Ser Cys Leu Lys Glu Glu  
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cataacataa ggaaaagcaa gcgtaatctt caggataatt ttcaggaga cccaatccag 300  
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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Vinkemeier, Uwe  
Darnell, Jr., James E.

(ii) TITLE OF INVENTION: PURIFIED STAT PROTEINS AND METHODS OF  
PURIFYING THEREOF

(iii) NUMBER OF SEQUENCES: 8

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: David A. Jackson, Esq.  
(B) STREET: 411 Hackensack Ave, Continental Plaza, 4th  
Floor  
(C) CITY: Hackensack  
(D) STATE: New Jersey  
(E) COUNTRY: USA  
(F) ZIP: 07601

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US  
(B) FILING DATE:  
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Jackson Esq., David A.  
(B) REGISTRATION NUMBER: 26,742  
(C) REFERENCE/DOCKET NUMBER: 600-1-182 N

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 201-487-5800  
(B) TELEFAX: 201-343-1684

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 750 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:  
(B) CLONE: Human Stat91

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Tyr Leu Ala Gln Trp Leu Glu Lys Gln Asp Trp Glu His Ala Ala Asn  
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Asp Val Ser Phe Ala Thr Ile Arg Phe His Asp Leu Leu Ser Gln Leu  
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Asp Asp Gln Tyr Ser Arg Phe Ser Leu Glu Asn Asn Phe Leu Leu Gln  
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His Asn Ile Arg Lys Ser Lys Arg Asn Leu Gln Asp Asn Phe Gln Glu  
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Asp Pro Ile Gln Met Ser Met Ile Ile Tyr Ser Cys Leu Lys Glu Glu  
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Arg Lys Ile Leu Glu Asn Ala Gln Arg Phe Asn Gln Ala Gln Ser Gly  
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 165 170 175

Leu Gln Asn Arg Glu His Glu Thr Asn Gly Val Ala Lys Ser Asp Gln  
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Glu Leu Thr Gln Asn Ala Leu Ile Asn Asp Glu Leu Val Glu Trp Lys  
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Arg Arg Gln Gln Ser Ala Cys Ile Gly Gly Pro Pro Asn Ala Cys Leu  
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Asp Gln Leu Gln Asn Trp Phe Thr Ile Val Ala Glu Ser Leu Gln Gln  
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Val Arg Gln Gln Leu Lys Lys Leu Glu Glu Leu Glu Gln Lys Tyr Thr  
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Tyr Glu His Asp Pro Ile Thr Lys Asn Lys Gln Val Leu Trp Asp Arg  
 290 295 300

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Ala Ala Glu Phe Arg His Leu Gln Leu Lys Glu Gln Lys Asn Ala Gly  
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425

430

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 Gln Leu Ser Glu Val Leu Ser Trp Gln Phe Ser Ser Val Thr Lys Arg  
 500 505 510  
 Gly Leu Asn Val Asp Gln Leu Asn Met Leu Gly Glu Lys Leu Leu Gly  
 515 520 525  
 Pro Asn Ala Ser Pro Asp Gly Leu Ile Pro Trp Thr Arg Phe Cys Lys  
 530 535 540  
 Glu Asn Ile Asn Asp Lys Asn Phe Pro Phe Trp Leu Trp Ile Glu Ser  
 545 550 555 560  
 Ile Leu Glu Leu Ile Lys Lys His Leu Leu Pro Leu Trp Asn Asp Gly  
 565 570 575  
 Cys Ile Met Gly Phe Ile Ser Lys Glu Arg Glu Arg Ala Leu Leu Lys  
 580 585 590  
 Asp Gln Gln Pro Gly Thr Phe Leu Leu Arg Phe Ser Glu Ser Ser Arg  
 595 600 605  
 Glu Gly Ala Ile Thr Phe Thr Trp Val Glu Arg Ser Gln Asn Gly Gly  
 610 615 620  
 Glu Pro Asp Phe His Ala Val Glu Pro Tyr Thr Lys Lys Glu Leu Ser  
 625 630 635 640  
 Ala Val Thr Phe Pro Asp Ile Ile Arg Asn Tyr Lys Val Met Ala Ala  
 645 650 655  
 Glu Asn Ile Pro Glu Asn Pro Leu Lys Tyr Leu Tyr Pro Asn Ile Asp  
 660 665 670  
 Lys Asp His Ala Phe Gly Lys Tyr Tyr Ser Arg Pro Lys Glu Ala Pro  
 675 680 685  
 Glu Pro Met Glu Leu Asp Gly Pro Lys Gly Thr Gly Tyr Ile Lys Thr  
 690 695 700  
 Glu Leu Ile Ser Val Ser Glu Val His Pro Ser Arg Leu Gln Thr Thr  
 705 710 715 720  
 Asp Asn Leu Leu Pro Met Ser Pro Glu Glu Phe Asp Glu Val Ser Arg  
 725 730 735  
 Ile Val Gly Ser Val Glu Phe Asp Ser Met Met Asn Thr Val  
 740 745 750

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 712 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Ser	Gln	Trp	Tyr	Glu	Leu	Gln	Leu	Asp	Ser	Lys	Phe	Leu	Glu	
1				5				10					15		
Gln	Val	His	Gln	Leu	Tyr	Asp	Asp	Ser	Phe	Pro	Met	Glu	Ile	Arg	Gln
	20					25							30		
Tyr	Leu	Ala	Gln	Trp	Leu	Glu	Lys	Gln	Asp	Trp	Glu	His	Ala	Ala	Asn
	35					40						45			
Asp	Val	Ser	Phe	Ala	Thr	Ile	Arg	Phe	His	Asp	Leu	Leu	Ser	Gln	Leu
	50					55					60				
Asp	Asp	Gln	Tyr	Ser	Arg	Phe	Ser	Leu	Glu	Asn	Asn	Phe	Leu	Leu	Gln
	65				70				75				80		
His	Asn	Ile	Arg	Lys	Ser	Lys	Arg	Asn	Leu	Gln	Asp	Asn	Phe	Gln	Glu
		85						90					95		
Asp	Pro	Ile	Gln	Met	Ser	Met	Ile	Ile	Tyr	Ser	Cys	Leu	Lys	Glu	Glu
		100					105					110			
Arg	Lys	Ile	Leu	Glu	Asn	Ala	Gln	Arg	Phe	Asn	Gln	Ala	Gln	Ser	Gly
	115						120					125			
Asn	Ile	Gln	Ser	Thr	Val	Met	Leu	Asp	Lys	Gln	Lys	Glu	Leu	Asp	Ser
	130					135					140				
Lys	Val	Arg	Asn	Val	Lys	Asp	Lys	Val	Met	Cys	Ile	Glu	His	Glu	Ile
	145				150				155				160		
Lys	Ser	Leu	Glu	Asp	Leu	Gln	Asp	Glu	Tyr	Asp	Phe	Lys	Cys	Lys	Thr
		165					170					175			
Leu	Gln	Asn	Arg	Glu	His	Glu	Thr	Asn	Gly	Val	Ala	Lys	Ser	Asp	Gln
		180					185					190			
Lys	Gln	Glu	Gln	Leu	Leu	Leu	Lys	Lys	Met	Tyr	Leu	Met	Leu	Asp	Asn
	195						200					205			
Lys	Arg	Lys	Glu	Val	Val	His	Lys	Ile	Ile	Glu	Leu	Leu	Asn	Val	Thr
	210					215					220				
Glu	Leu	Thr	Gln	Asn	Ala	Leu	Ile	Asn	Asp	Glu	Leu	Val	Glu	Trp	Lys
	225					230					235			240	
Arg	Arg	Gln	Gln	Ser	Ala	Cys	Ile	Gly	Gly	Pro	Pro	Asn	Ala	Cys	Leu
		245					250					255			
Asp	Gln	Leu	Gln	Asn	Trp	Phe	Thr	Ile	Val	Ala	Glu	Ser	Leu	Gln	Gln
		260					265					270			
Val	Arg	Gln	Gln	Leu	Lys	Lys	Leu	Glu	Leu	Glu	Gln	Lys	Tyr	Thr	
		275					280					285			
Tyr	Glu	His	Asp	Pro	Ile	Thr	Lys	Asn	Lys	Gln	Val	Leu	Trp	Asp	Arg
	290					295					300				
Thr	Phe	Ser	Leu	Phe	Gln	Gln	Leu	Ile	Gln	Ser	Ser	Phe	Val	Val	Glu
	305						310				315			320	

Arg Gln Pro Cys Met Pro Thr His Pro Gln Arg Pro Leu Val Leu Lys  
 325 330 335  
 Thr Gly Val Gln Phe Thr Val Lys Leu Arg Leu Leu Val Lys Leu Gln  
 340 345 350  
 Glu Leu Asn Tyr Asn Leu Lys Val Lys Val Leu Phe Asp Lys Asp Val  
 355 360 365  
 Asn Glu Arg Asn Thr Val Lys Gly Phe Arg Lys Phe Asn Ile Leu Gly  
 370 375 380  
 Thr His Thr Lys Val Met Asn Met Glu Glu Ser Thr Asn Gly Ser Leu  
 385 390 395 400  
 Ala Ala Glu Phe Arg His Leu Gln Leu Lys Glu Gln Lys Asn Ala Gly  
 405 410 415  
 Thr Arg Thr Asn Glu Gly Pro Leu Ile Val Thr Glu Glu Leu His Ser  
 420 425 430  
 Leu Ser Phe Glu Thr Gln Leu Cys Gln Pro Gly Leu Val Ile Asp Leu  
 435 440 445  
 Glu Thr Thr Ser Leu Pro Val Val Val Ile Ser Asn Val Ser Gln Leu  
 450 455 460  
 Pro Ser Gly Trp Ala Ser Ile Leu Trp Tyr Asn Met Leu Val Ala Glu  
 465 470 475 480  
 Pro Arg Asn Leu Ser Phe Phe Leu Thr Pro Pro Cys Ala Arg Trp Ala  
 485 490 495  
 Gln Leu Ser Glu Val Leu Ser Trp Gln Phe Ser Ser Val Thr Lys Arg  
 500 505 510  
 Gly Leu Asn Val Asp Gln Leu Asn Met Leu Gly Glu Lys Leu Leu Gly  
 515 520 525  
 Pro Asn Ala Ser Pro Asp Gly Leu Ile Pro Trp Thr Arg Phe Cys Lys  
 530 535 540  
 Glu Asn Ile Asn Asp Lys Asn Phe Pro Phe Trp Leu Trp Ile Glu Ser  
 545 550 555 560  
 Ile Leu Glu Leu Ile Lys Lys His Leu Leu Pro Leu Trp Asn Asp Gly  
 565 570 575  
 Cys Ile Met Gly Phe Ile Ser Lys Glu Arg Glu Arg Ala Leu Leu Lys  
 580 585 590  
 Asp Gln Gln Pro Gly Thr Phe Leu Leu Arg Phe Ser Glu Ser Ser Arg  
 595 600 605  
 Glu Gly Ala Ile Thr Phe Thr Trp Val Glu Arg Ser Gln Asn Gly Gly  
 610 615 620  
 Glu Pro Asp Phe His Ala Val Glu Pro Tyr Thr Lys Lys Glu Leu Ser  
 625 630 635 640  
 Ala Val Thr Phe Pro Asp Ile Ile Arg Asn Tyr Lys Val Met Ala Ala  
 645 650 655  
 Glu Asn Ile Pro Glu Asn Pro Leu Lys Tyr Leu Tyr Pro Asn Ile Asp  
 660 665 670  
 Lys Asp His Ala Phe Gly Lys Tyr Tyr Ser Arg Pro Lys Glu Ala Pro

675

680

685

Glu Pro Met Glu Leu Asp Gly Pro Lys Gly Thr Gly Tyr Ile Lys Thr  
 690                    695                    700

Glu Leu Ile Ser Val Ser Glu Val  
 705                    710

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 582 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein

- (iii) HYPOTHETICAL: NO

- (v) FRAGMENT TYPE: internal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ser Thr Val Met Leu Asp Lys Gln Lys Glu Leu Asp Ser Lys Val Arg  
 1                    5                    10                    15

Asn Val Lys Asp Lys Val Met Cys Ile Glu His Glu Ile Lys Ser Leu  
 20                    25                    30

Glu Asp Leu Gln Asp Glu Tyr Asp Phe Lys Cys Lys Thr Leu Gln Asn  
 35                    40                    45

Arg Glu His Glu Thr Asn Gly Val Ala Lys Ser Asp Gln Lys Gln Glu  
 50                    55                    60

Gln Leu Leu Leu Lys Lys Met Tyr Leu Met Leu Asp Asn Lys Arg Lys  
 65                    70                    75                    80

Glu Val Val His Lys Ile Ile Glu Leu Leu Asn Val Thr Glu Leu Thr  
 85                    90                    95

Gln Asn Ala Leu Ile Asn Asp Glu Leu Val Glu Trp Lys Arg Arg Gln  
 100                    105                    110

Gln Ser Ala Cys Ile Gly Gly Pro Pro Asn Ala Cys Leu Asp Gln Leu  
 115                    120                    125

Gln Asn Trp Phe Thr Ile Val Ala Glu Ser Leu Gln Gln Val Arg Gln  
 130                    135                    140

Gln Leu Lys Lys Leu Glu Glu Leu Glu Gln Lys Tyr Thr Tyr Glu His  
 145                    150                    155                    160

Asp Pro Ile Thr Lys Asn Lys Gln Val Leu Trp Asp Arg Thr Phe Ser  
 165                    170                    175

Leu Phe Gln Gln Leu Ile Gln Ser Ser Phe Val Val Glu Arg Gln Pro  
 180                    185                    190

Cys Met Pro Thr His Pro Gln Arg Pro Leu Val Leu Lys Thr Gly Val  
 195                    200                    205

Gln Phe Thr Val Lys Leu Arg Leu Leu Val Lys Leu Gln Glu Leu Asn  
 210 215 220  
 Tyr Asn Leu Lys Val Lys Val Leu Phe Asp Lys Asp Val Asn Glu Arg  
 225 230 235 240  
 Asn Thr Val Lys Gly Phe Arg Lys Phe Asn Ile Leu Gly Thr His Thr  
 245 250 255  
 Lys Val Met Asn Met Glu Glu Ser Thr Asn Gly Ser Leu Ala Ala Glu  
 260 265 270  
 Phe Arg His Leu Gln Leu Lys Glu Gln Lys Asn Ala Gly Thr Arg Thr  
 275 280 285  
 Asn Glu Gly Pro Leu Ile Val Thr Glu Glu Leu His Ser Leu Ser Phe  
 290 295 300  
 Glu Thr Gln Leu Cys Gln Pro Gly Leu Val Ile Asp Leu Glu Thr Thr  
 305 310 315 320  
 Ser Leu Pro Val Val Val Ile Ser Asn Val Ser Gln Leu Pro Ser Gly  
 325 330 335  
 Trp Ala Ser Ile Leu Trp Tyr Asn Met Leu Val Ala Glu Pro Arg Asn  
 340 345 350  
 Leu Ser Phe Phe Leu Thr Pro Pro Cys Ala Arg Trp Ala Gln Leu Ser  
 355 360 365  
 Glu Val Leu Ser Trp Gln Phe Ser Ser Val Thr Lys Arg Gly Leu Asn  
 370 375 380  
 Val Asp Gln Leu Asn Met Leu Gly Glu Lys Leu Leu Gly Pro Asn Ala  
 385 390 395 400  
 Ser Pro Asp Gly Leu Ile Pro Trp Thr Arg Phe Cys Lys Glu Asn Ile  
 405 410 415  
 Asn Asp Lys Asn Phe Pro Phe Trp Leu Trp Ile Glu Ser Ile Leu Glu  
 420 425 430  
 Leu Ile Lys Lys His Leu Leu Pro Leu Trp Asn Asp Gly Cys Ile Met  
 435 440 445  
 Gly Phe Ile Ser Lys Glu Arg Glu Arg Ala Leu Leu Lys Asp Gln Gln  
 450 455 460  
 Pro Gly Thr Phe Leu Leu Arg Phe Ser Glu Ser Ser Arg Glu Gly Ala  
 465 470 475 480  
 Ile Thr Phe Thr Trp Val Glu Arg Ser Gln Asn Gly Gly Glu Pro Asp  
 485 490 495  
 Phe His Ala Val Glu Pro Tyr Thr Lys Lys Glu Leu Ser Ala Val Thr  
 500 505 510  
 Phe Pro Asp Ile Ile Arg Asn Tyr Lys Val Met Ala Ala Glu Asn Ile  
 515 520 525  
 Pro Glu Asn Pro Leu Lys Tyr Leu Tyr Pro Asn Ile Asp Lys Asp His  
 530 535 540  
 Ala Phe Gly Lys Tyr Tyr Ser Arg Pro Lys Glu Ala Pro Glu Pro Met  
 545 550 555 560  
 Glu Leu Asp Gly Pro Lys Gly Thr Gly Tyr Ile Lys Thr Glu Leu Ile

565

570

575

Ser Val Ser Glu Val His  
580

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 131 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (v) FRAGMENT TYPE: N-terminal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met	Ser	Gln	Trp	Tyr	Glu	Leu	Gln	Gln	Leu	Asp	Ser	Lys	Phe	Leu	Glu
1					5				10					15	
Gln	Val	His	Gln	Leu	Tyr	Asp	Asp	Ser	Phe	Pro	Met	Glu	Ile	Arg	Gln
				20				25					30		
Tyr	Leu	Ala	Gln	Trp	Leu	Glu	Lys	Gln	Asp	Trp	Glu	His	Ala	Ala	Asn
				35			40					45			
Asp	Val	Ser	Phe	Ala	Thr	Ile	Arg	Phe	His	Asp	Leu	Leu	Ser	Gln	Leu
	50					55					60				
Asp	Asp	Gln	Tyr	Ser	Arg	Phe	Ser	Leu	Glu	Asn	Asn	Phe	Leu	Gln	
	65				70				75			80			
His	Asn	Ile	Arg	Lys	Ser	Lys	Arg	Asn	Leu	Gln	Asp	Asn	Phe	Gln	Glu
				85				90				95			
Asp	Pro	Ile	Gln	Met	Ser	Met	Ile	Ile	Tyr	Ser	Cys	Leu	Lys	Glu	Glu
				100				105				110			
Arg	Lys	Ile	Leu	Glu	Asn	Ala	Gln	Arg	Phe	Asn	Gln	Ala	Gln	Ser	Gly
				115				120				125			
Asn	Ile	Gln													
	130														

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1746 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGCACAGTGA	TGTTAGACAA	ACAGAAAGAG	CTTGACAGTA	AAGTCAGAAA	TGTGAAGGAC	60
AAGGTTATGT	GTATAGAGCA	TGAAATCAAG	AGCCTGGAAG	ATTTACAAGA	TGAATATGAC	120
TTCAAATGCA	AAACCTTGCA	GAACAGAGAA	CACGAGACCA	ATGGTGTGGC	AAAGAGTGAT	180
CAGAAACAAG	AACAGCTGTT	ACTCAAGAAG	ATGTATTTAA	TGCTTGACAA	TAAGAGAAAG	240
GAAGTAGTTC	ACAAAATAAT	AGAGTTGCTG	AATGTCACTG	AACTTACCCA	GAATGCCCTG	300
ATTAATGATG	AACTAGTGGA	GTGGAAGCGG	AGACAGCAGA	GCGCCTGTAT	TGGGGGGCCG	360
CCCAATGCTT	GCTTGGATCA	GCTGCAGAAC	TGGTTCACTA	TAGTTGCGGA	GAGTCTGCAG	420
CAAGTTCGGC	AGCAGCTTAA	AAAGTTGGAG	GAATTGGAAC	AGAAATACAC	CTACGAACAT	480
GACCCTATCA	CAAAAAACAA	ACAAGTGT TA	TGGGACCGCA	CCTTCAGTCT	TTTCCAGCAG	540
CTCATTCA G	GCTCGTTGT	GGTGGAAAGA	CAGCCCTGCA	TGQCAACGCA	CCCTCAGAGG	600
CCGCTGGTCT	TGAAGACAGG	GGTCCAGTTC	ACTGTGAAGT	TGAGACTGTT	GGTGAATTG	660
CAAGAGCTGA	ATTATAATT	GAAAGTCAAA	GTCTTATTTG	ATAAAGATGT	GAATGAGAGA	720
AATACAGTAA	AAGGATT TAG	GAAGTTCAAC	ATTTGGC A	CGCACACAAA	AGTGATGAAC	780
ATGGAGGAGT	CCACCAATGG	CAGTCTGGCG	GCTGAATTTC	GGCACCTGCA	ATTGAAAGAA	840
CAGAAAAATG	CTGGCACCA G	AACGAATGAG	GGTCCTCTCA	TCGTTACTGA	AGAGCTTCAC	900
TCCCTTAGTT	TTGAAACCCA	ATTGTGCCAG	CCTGGTTTGG	TAATTGACCT	CGAGACGACC	960
TCTCTGCCG	TTGTGGTGAT	CTCCAACGTC	AGCCAGCTCC	CGAGCGGTTG	GGCCTCCATC	1020
CTTTGGTACA	ACATGCTGGT	GGCGGAACCC	AGGAATCTGT	CCTCTTCCCT	GACTCCACCA	1080
TGTGCACGAT	GGGCTCAGCT	TTCAGAAGTG	CTGAGTTGGC	AGTTTCTTC	TGTCACCAAA	1140
AGAGGTCTCA	ATGTGGACCA	GCTGAACATG	TTGGGAGAGA	AGCTTCTTGG	TCCTAACGCC	1200
AGCCCCGATG	GTCTCATTCC	GTGGACGAGG	TTTTGTAAGG	AAAATATAAA	TGATAAAAAT	1260
TTTCCCTTCT	GGCTTGAT	TGAAAGCATC	CTAGAACTCA	TTAAAAAAACA	CCTGCTCCCT	1320
CTCTGGAAATG	ATGGGTGCAT	CATGGGCTTC	ATCAGCAAGG	AGCGAGAGCG	TGCCCTGTTG	1380
AAGGACCAGC	AGCCGGGGAC	CTTCCTGCTG	CGGTTCA GTG	AGAGCTCCCG	GGAGGGGCC	1440
ATCACATTCA	CATGGGTGGA	GCGGTCCCAG	AACGGAGGCG	AACTGACTT	CCATGCGGTT	1500
GAACCTACA	CGAAGAAAGA	ACTTTCTGCT	GTTACTTTCC	CTGACATCAT	TCGCAATTAC	1560
AAAGTCATGG	CTGCTGAGAA	TATTCCTGAG	AATCCCCTGA	AGTATCTGTA	TCCAAATATT	1620
GACAAAGACC	ATGCCTTGG	AAAGTATTAC	TCCAGGCCAA	AGGAAGCACC	AGAGCCAATG	1680
GAACCTGATG	GCCCTAAAGG	AACTGGATAT	ATCAAGACTG	AGTTGATTTC	TGTGTCTGAA	1740
GTTCAC						1746

## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 393 base pairs
  - (B) TYPE: nucleic acid

(C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATGTCTCACT GGTACGAACT TCAGCAGCTT GACTCAAAAT TCCTGGAGCA GGTTCACCAAG	60
CTTTATGATG ACAGTTTCC CATGGAAATC AGACAGTACC TGGCACAGTG GTTAGAAAAG	120
CAAGACTGGG AGCACGCTGC CAATGATGTT TCATTTGCCA CCATCCGTTT TCATGACCTC	180
CTGTCACAGC TGGATGATCA ATATACTCGC TTTTCTTGG AGAATAACTT CTTGCTACAG	240
CATAAACATAA GGAAAAGCAA GCGTAATCTT CAGGATAATT TTCAGGAAGA CCCAATCCAG	300
ATGTCTATGA TCATTTACAG CTGCTGAAG GAAGAAAGGA AAATTCTGGA AAACGCCAG	360
AGATTAAATC AGGCTCAGTC GGGGAATATT CAG	393

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 36 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
 (A) DESCRIPTION: /desc = "Primer"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGGAATTCCA TATGAGCACACA GTGATGTTAG ACAAAC	36
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(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 34 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
 (A) DESCRIPTION: /desc = "Primer"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGGATCCTAT TAGTGAACCTT CAGACACAGA AATC	34
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PATENT  
600-1-182NA

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT : UWE VINKEMEIER *ET AL.*

SERIAL NO. : UNASSIGNED EXAMINER : UNKNOWN

FILED : HEREWITH ART UNIT : UNKNOWN

FOR : PURIFIED STAT PROTEINS AND METHODS OF PURIFYING THEREOF

REQUEST FOR TRANSFER OF COMPUTER READABLE FORM

ASSISTANT COMMISSIONER FOR PATENTS  
WASHINGTON, D.C. 20231

Sir:

Applicants request that the previously submitted sequence information filed in computer readable form in Application Number 08/951,130 be used in the present Application. This sequence information was the second computer readable form filed in that Application.

Respectfully submitted,

  
MICHAEL D. DAVIS  
Attorney for Applicant(s)  
Registration No. 39,161

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Date: November 2, 1999